membranes. HeLa cells transiently transfected with cytoplasmic S-F_v2-Casp3-E (C), plasma membrane-localized M-F_v2-Casp3-E (D), mitochondria-localized $Mas70_{34}$-F_v2-Casp3-E (E), or nuclear N2-F_v2-Casp3-E (F) were fixed, stained with anti-HA antibodies and examined by confocal microscopy. Alternatively, control proteins were localized, including S-F_v2-E (G), M-F_v2-E (H), Bcl-x_L-E (I), or Gal4-VP16-E (J). In each case, cells shown are representative of several transfected cells.

Figure 27. Nuclear-targeted caspase-1, -3, and -8 trigger apoptosis. (A-B) Transient transfection assay was performed as above. (A and B) Cells received 2 μg SRα-SEAP plus 2 μg nuclear targeted caspases, including N2-F_v1-F_{vls}1-Casp1 (Δ), N2-F_v1-F_{vls}1-Casp3 (▲), N2-F_v1-F_{vls}1-Casp8 (▣), or control construct N2-F_v1-F_{vls}1 (□). (C) Nuclear targeted caspase-3 functions efficiently. Cells received SRα-SEAP plus 4 μg (□) or 1 μg (Δ) S-F_v2-Casp3, 4 μg (▣) or 1 μg (▲) N2-F_v2-Casp, or 1 μg S-F_v1-F_{vls}1 (○). (D) Nuclear targeted $FADD_{125}$ has reduced activity. Cells received reporter plasmid plus 2 μg S-F_v1-F_{vls}1-$FADD_{125}$ (▣),N2-F_v1-F_{vls}1-$FADD_{125}$ (□), or N2-F_v1-F_{vls}1 (Δ).

Figure 28. A) miniprep gel of pAdTrack-CMV (9220bp); B) miniprep gel of pAdTrack-CMV, pSH1/S-E-F_v1-F_{vls}1-ICEst and pSH1/S-F_{vls}1-Yama-E;

Figure 29. A) Representation of pAdTrack-CMV; B) and C) Gel of miniprep check of pAdTrack-CMV-S-F_{vls}1-Yama-E .

Figure 30. A) Representation of pAdTrack-CMV; B), C) and D) Gel of miniprep check of pAdTrack-CMV-S-F_v1-F_{vls}1-ICEst.

Figure 31. A) Gel of miniprep check of pSH1/S-E-F_v1-F_{vls}1-ICEst and B) Representation of pSH1/S-E-F_v1-F_{vls}1-ICEst;

Figure 32. Representation of pSH1/S-F_{vls}1-Yama-E;

Figure 33. Outline of the Generation of ADV-GFP-CMV-Yama-E and ADV-GFP-CMV-E-ICE;

Figure 34. Schematic diagram showing general protocol for generation of ADV-GFP-CMV-Yama-E and ADV-GFP-CMV-E-ICE;

Figure 35. A) Representation of pADEasy-1 and B) Gel of miniprep check of pADEasy-1;

Figure 36. A) Gel of miniprep check of pADEasy-1-Track-CMV-E-ICE; B) and C) Gels of miniprep checks of pAdEasy-1-Track-CMV-Yama-E;

Figure 37. A) miniprep gel check of colony #2 of pShuttle-CMV-E-F_v1-F_{vls}1-ICEst; B) miniprep gel check of pShuttle-CMV-E-F_v1-F_{vls}1-ICEst from multiple colonies of transformed E.coli BJ5183 competent cells;

Figure 38. Schematic representation of protocol forADV-CMV-E-ICE;

Figure 39. A) Representation of pShuttle-CMV and B) miniprep gel;

Figure 40. Results of Luciferase assay of pShuttle-CMV-F_v1-F_{vls}1-ICE-E;

Figure 41. Outline of Assay of Effect of Ad-YAMA and Ad-ICE on Different cell types;

Figure 42. Graph showing effect of Ad-YAMA and Ad-ICE on T-C2G cells;

Figure 43. Graph showing effect of Ad-YAMA and Ad-ICE on T-C2 cells;

Figure 44. Graph showing effect of Ad-YAMA and Ad-ICE on JD-2a cells;

Figure 45. Graph showing effect of Ad-YAMA and Ad-ICE on LNCaP cells;

Figure 46. Outline and Western Blot showing expression and activation of ICE and YAMA;

Figure 47. A) untreated JD-2α cell culture; B) control culture incubated with Adv-Fv1-YAMA expressing green fluorescent protein; C) cell culture incubated with virus and maintained in 50nM AP1903;

Figure 48. Plated PC-3 cells incubated with ADV-FKBP/ICE and treated (+) or untreated with AP1903 at increasing MOI;.

Figure 49. Plated JD-2a BPH cells incubated with ADV-FKBP/ICE and treated (+) or untreated with AP1903 at increasing MOI;

Figure 50. Diagram illustrating protocol for treatment of s.c. prostate adenocarcinoma in situ with CID inducible caspases;

EXAMPLE 17

Construction of an adenovirus expressing a chemically inducible apoptosis factor.

The E-F_v2 YAMA-E construct described previously was placed under the control of the CMV promoter and inserted into an adenoviral recombination vector. The adenoviral recombination vector contained a copy of the D1 gene from adenovirus. The CMV promoter - E-F_v2-YAMA-E construct was inserted such that the F_v2 construct was flanked on both sides by nucleotide sequences from the E1 gene.

The plasmid was transfected into adenovirus infected cells and a recombinant adenovirus expressing the chemically inducible apoptosis factor was isolated and purified by standard methods.

Figure 19 A shows a plasmid map of the adenoviral recombination vector used to construct an adenovirus expressing E-F_V2-YAMA-E under control of the CMV promoter and incorporating the 16S splice junction to improve the efficiency of mRNA processing (Takabe, *et al.* Mol. Cell. Bio. 8:466-472, 1988). Panel B slows the results of a restriction analysis of the plasmid. Figure 20 A shows a plasmid map of the plasmid used to construct a recombinant adenovirus expressing E-F_V2-YAMA-E under the control of the SRα promoter. Panel B shows the results of a restriction analysis of the plasmid.

EXAMPLE 18

Construction of Additional Adenovirus Constructs Containing Conditional Caspase 1 or 3

I. Construction of pAdTrack-CMV-F_{vls}1-Yama-E (Fig. 28)

1. Digest pAdTrack-CMV (Fig. 29A) with Eco RV and Not I. Purify the 9.2 kb vector by agarose electrophoresis and GeneClean (Fig. 28 B).
2. Digest pSH1/S-F_{vls}1-Yama-E (Fig. 32) with Eco RI and Not I. Blunt the Eco RI end. Purify the 1.2 kb fragment by agarose electrophoresis and GeneClean.
3. Ligate the above two fragments and transform XL-1 Blue with the ligation. Chose several colonies, do Miniprep and check with Sal I, Not I + Eco RI, Hind III + Eco RI, Hind III + Xho I. See Figs. 29 B and 29 C.

II. Construction of pAdTrack-CMV-E-F_v1-$F_{vls}1$-ICEst (See Fig. 28)

1. Digest pAdTrack-CMV with Eco RV and Not I. Purify the 9.2 kb vector by agarose electrophoresis and GeneClean (Fig. 28 B).
2. Digest pSH1/S-E-F_v1-$F_{vls}1$-ICEst (Fig. 31 B) with Eco RI and Not I. Blunt the Eco RI end. Purify the 2.2 kb fragment by agarose electrophoresis and GeneClean.
3. Ligate the above two fragments and transform XL-1 Blue with the ligation. Chose several colonies, do Miniprep and check with Sal I, Not I + Eco RI, Hind III + Eco RI, Hind III + Xho I. See Figs. 30 B through 30 D.

III. Generation of ADV-GFP-CMV-Yama-E (Figs. 33 and 34)

1. Linearize 1 μg pAdTrack-CMV-$F_{vls}1$-Yama-E with Pme I. Purify it by phenol-chloroform extraction, ethanol precipitation and resuspend in 6 μl H2O.
2. Mix it with 100 μg p AdEasy-1 (Fig. 35) (in 1 μl), Co-transform 20 μl E. coli BJ5183 competent cells with GenePulser at 2,500 V, 200 Ohms, 25 uFD.
3. Pick up 20 smallest colonies. Do Miniprep and check with Pac I. Candidate clones usually yield a large fragment (near 30 kb), plus a smaller fragment of 3.0 kb or 4.5 kb.
4. Re-transform the correct recombinant plasmids into XL-1 Blue. Midiprep with Qiagen kit.
5. Transfect 293 cell by the recombinant plasmid with FuGene. 4μg DNA/6μl FuGene/well (6 well-plate). Check GFP expression with fluorescent microscope.
6. Harvest the cells when 30% of them are detached. Spin down the cells; use the supernatant for next infection. Repeat infection for several rounds.
7. Collect the cells, repeat freeze/thaw/vortex four times. Purify the virus by CsCl gradient centrifuge.

IV. Generation of ADV-GFP-CMV-E-ICE (Figs. 33 and 34)

1. Linearize 1 μg pAdTrack-CMV- E-F_v1-F_{vls}1-ICEst with Pme I. Purify it by phenol-chloroform extraction, ethanol precipitation and resuspend in 6 μl H_2O.
2. Mix it with 100 μg p AdEasy-1 (Fig. 35 A) (in 1 μl), Co-transform 20 μl E. coli BJ5183 competent cells with GenePulser at 2,500 V, 200 Ohms, 25 uFD.
3. Pick up 20 smallest colonies. Do miniprep and check with Pac I. Candidate clones usually yield a large fragment (near 30 kb), plus a smaller fragment of 3.0 kb or 4.5 kb.
4. Re-transform the correct recombinant plasmids into XL-1 Blue. Midiprep with Qiagen kit.
5. Transfect 293 cell by the recombinant plasmid with FuGene. 4μg DNA/6μl FuGene/well (6 well plate). Check GFP expression with fluorescent microscope.
6. Harvest the cells when 30% of them are detached. Spin down the cells, use the supernatant for next infection. Repeat infection for several rounds.
7. Collect the cells, repeat freeze/thaw/vortex four times. Purify the virus by CsCl gradient centrifuge.

V. Construction of pShuttle-CMV-E-F_v1-F_{vls}1-ICEst (Figs. 37-39)

1. Digest pShuttle-CMV (Fig. 39) with Eco RV and Not I. Purify the 7.4 kb vector by agarose electrophoresis and GeneClean.
2. Digest pSH1/S-E-F_v1-F_{vls}1-ICEst with Eco RI and Not I. Blunt the Eco RI end. Purify the 2.2 kb fragment by agarose electrophoresis and GeneClean. Fig. 38.
3. Ligate the above two fragments and transform XL-1 Blue with the ligation. Chose several colonies, do miniprep and check with Sal I and Eco RI.

VI. Generation of ADV-CMV-E-ICE (Figs. 37-39)

EXAMPLE 20

Assay To Determine Effects of Using Different Viruses (Figs. 41-45)

(See Table 4 for a more detailed protocol).

1. Cells were plated in 24 well-plate, $2X10^4$ to $4X10^4$ cells/well in 1 ml media (RPMI 1640 for JD-2α, LNCaP and PC3 cells; DMEM for Tramp, T-C2 and T-C2G cells) with 5% FBS, and incubated until the cell number doubled.
2. Cells were infected with the virus at different MOI and incubated overnight.
3. AP1903, or FK 1012,or AP20187 was added at a final concentration of 50 nM and cultures were incubated 24 hours.
4. Cells were fixed with 1% glutaraldehyde for 15 min.; stained with 0.5% crystal violet for 20 min.; washed with H2O for 30 min.; air dried; resolved with 200-500 ul/well Soreson's Solution for 5 min.; transfered 60—100 μl to each well of 96-well plate and read OD at 570 nm. Results are shown in Figs 42-45.

EXAMPLE 21

Replication deficient (Δ E1) adenoviral vectors expressing green flourescent protein and conditional Caspase 1 (ICE) or Caspase 3 (YAMA) were engineered. These vectors independently express green fluorescent protein so that infected cells are easily identified by their green color under fluorescent microscopy. These vectors were tested for their ability to induce apoptosis *in vitro* in a SMC line derived from a patient with BPH upon administration of a non-toxic, lipid-permeable, divalent FK506 analog (AP1903).

40,000 JD-2a cells per well were plated in 24-well plates and infected at a multiplicity-of-infection (MOI) of ~25 with Adv-F_V1-YAMA, an adenoviral vector expressing CID-regulated YAMA. After 24 hours, culture media was changed to control media ± 50nM AP1903 for an additional 24 hours, and the cells were viewed under fluorescent microscopy. All of the cells incubated with virus and maintained in control media appeared green, and were clearly attached and viable, similar to non-fluorescent

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What is claimed is:

1. A conditionally lethal molecule comprising a chemical inducer binding domain and an apoptosis inducing factor, wherein said apoptosis inducing factor is an apoptosis signal transducing factor.

2. A conditionally lethal molecule according to claim 1, wherein said apoptosis inducing factor is an adaptor molecule.

3. A conditionally lethal molecule according to claim 1, wherein said apoptosis inducing factor is a protease.

4. A conditionally lethal molecule according to claim 1, wherein said apoptosis inducing factor is a caspase.

5. A nucleic acid molecule encoding the conditionally lethal molecule of any one of claims 1-4.

6. A nucleic acid molecule according to claim 5, further comprising a sequence coding for tissue specific expression operatively linked to a sequence coding for a conditionally lethal molecule.

7. A gene therapy vector comprising a nucleic acid sequence coding for the expression of a conditionally lethal molecule according to anyone of claims 1-4.

8. A gene therapy vector according to claim 7, further comprising a sequence coding for a therapeutic gene.

9. A gene therapy vector according to claim 7, further comprising a sequence coding for tissue specific expression operatively linked to a sequence coding for a conditionally lethal molecule.

10. A transgenic animal expressing a conditionally lethal molecule according to any one of claims 1-4.

11. A method of making a transgenic animal comprising the step of micro-injecting a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4.

12. A method of treating a disease comprising the step of administering a vector that encodes a conditionally lethal molecule according to any one of claims 1-4.

13. A method according to claim 12, wherein the disease is a hyperproliferative disease.

14. A method according to claim 13, wherein the hyperproliferative disease is a benign disease.

15. A method according to claim 14, wherein the disease is a malignant disease.

16. A method according to claim 12, wherein the disease is atherosclerosis.

17. A method of causing regression of a tumor comprising transfecting cells of said tumor with a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4.

18. A method according to claim 17 further comprising administering a CID.

19. A method of causing a reduction in tumor size comprising transfecting cells of said tumor with a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4.

20. A method according to claim 19 further comprising administering a CID.

21. A method of causing a reduction in PSA levels comprising transfecting cells of a tumor with a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4.

22. A method according to claim 21 further comprising administering a CID.

23. A method of affecting the rate of cell proliferation caused by BPH comprising transfecting prostate cells with a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4.

24. A method according to claim 23 further comprising administering a CID.

25. A method of inducing apoptosis in a cell comprising transfecting said cell with a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4.

26. A method according to claim 26 further comprising administering a CID.

27. A method for determining the biological role of a cell type, comprising transfecting a cell of said cell type with a nucleic acid molecule encoding a conditionally lethal molecule according to any one of claims 1-4 and administering a CID.

ABSTRACT OF THE DISCLOSURE

The present invention discloses artificial death switches (ADSs) based on chemically induced dimerization of the cysteine proteases, caspase-1 (ICE) and caspase-3 (YAMA). In both cases, aggregation of the target protein is achieved by a non-toxic, lipid-permeable, dimeric FK506 analog that binds to an attached FK506-binding protein (FKBP). The intracellular crosslinking of caspase-1 or caspase-3 is sufficient to trigger rapid apoptosis in a Bcl-x_L-independent manner, suggesting that these conditional pro-apoptotic molecules can bypass intracellular checkpoint genes, like Bcl-x_L, that limit apoptosis. Since these chimeric molecules are derived from autologous proteins, they should be non-immunogenic and thus ideal for long-lived gene therapy vectors. These properties should also make chemically-induced apoptosis (CIA) useful for developmental studies, for treating hyperproliferative disorders and for developing animal models to a wide variety of diseases.

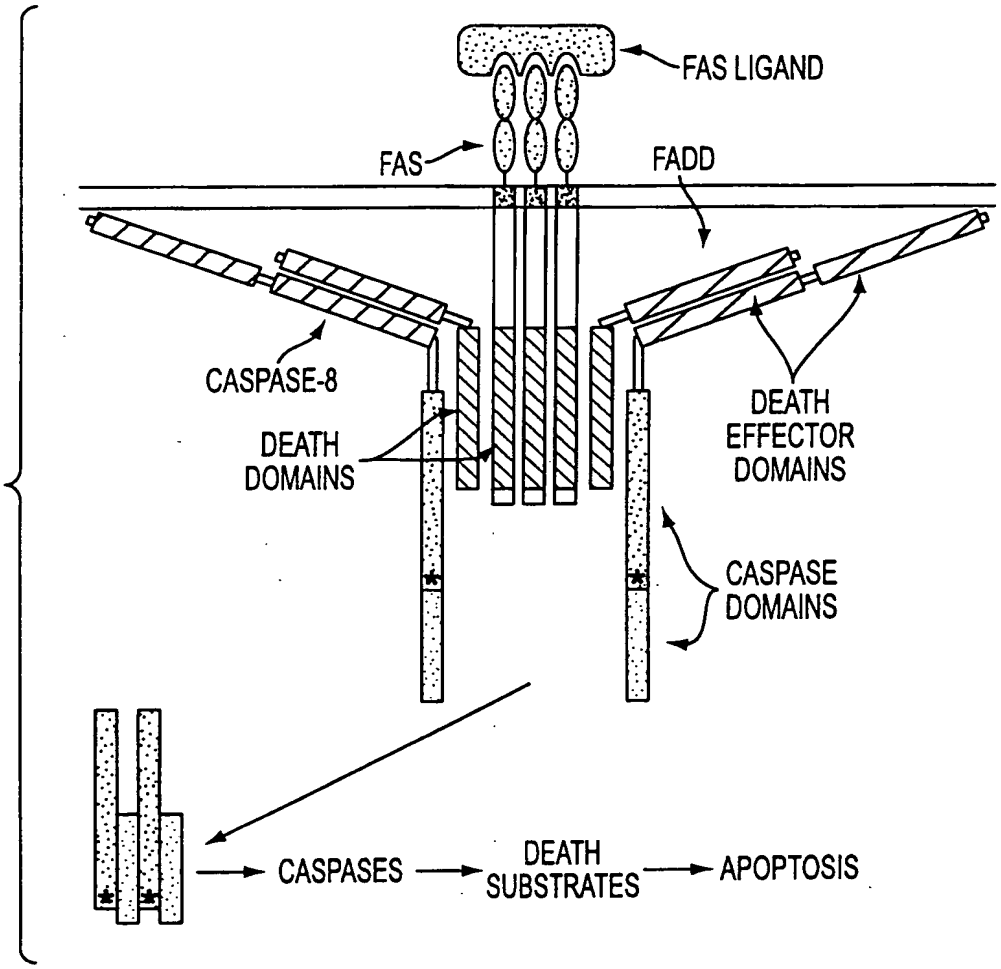


FIG. 1

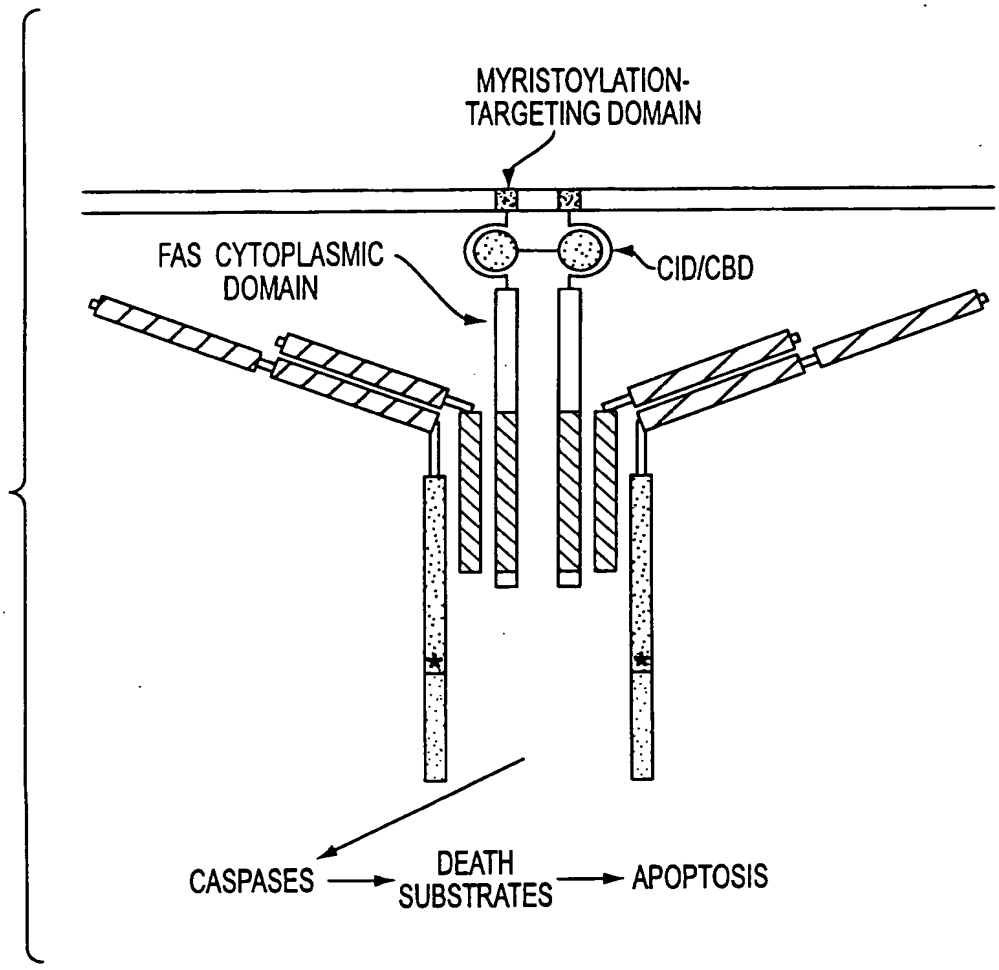


FIG. 2

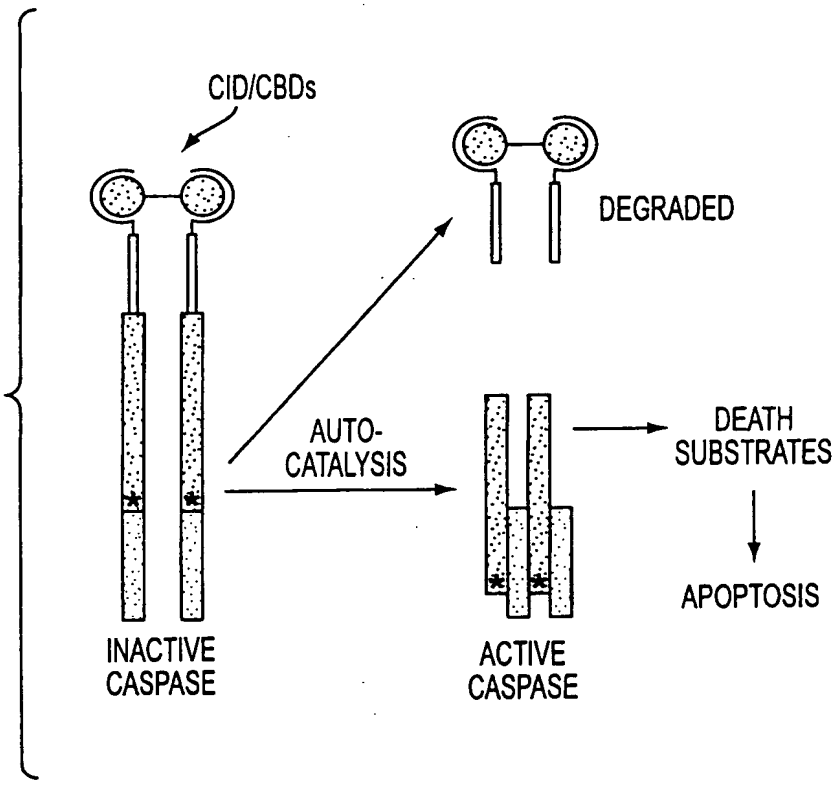


FIG. 3

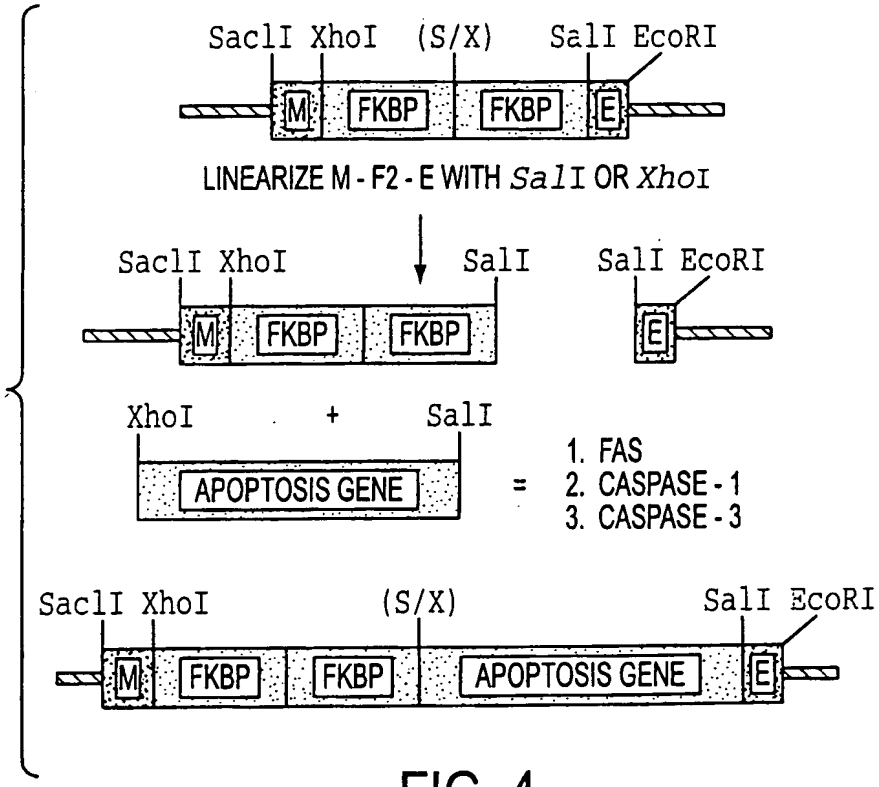
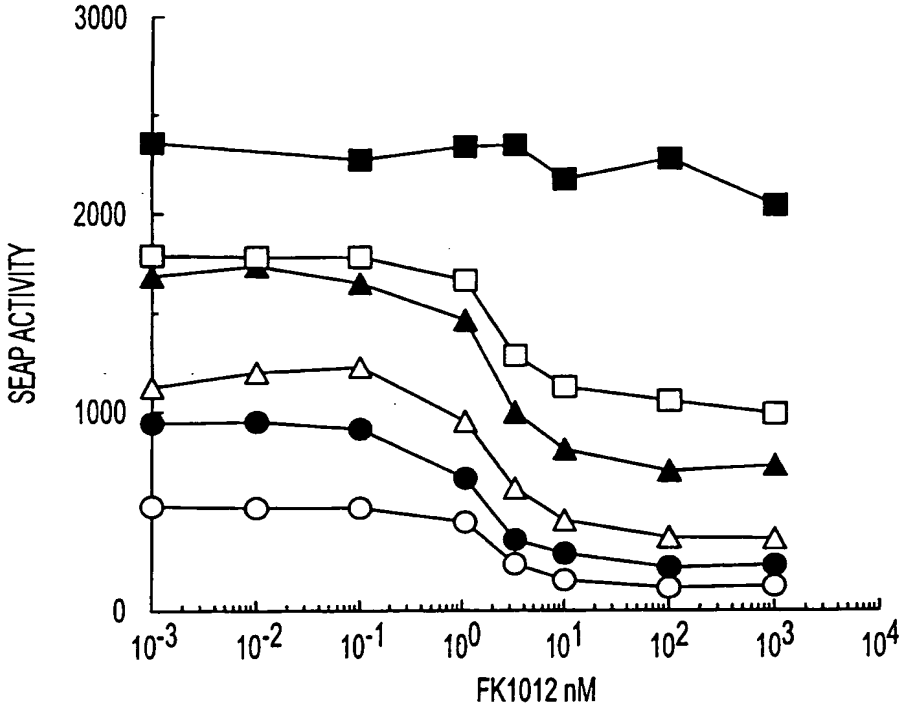


FIG. 4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2000
1000
0
SEAP ACTIVITY
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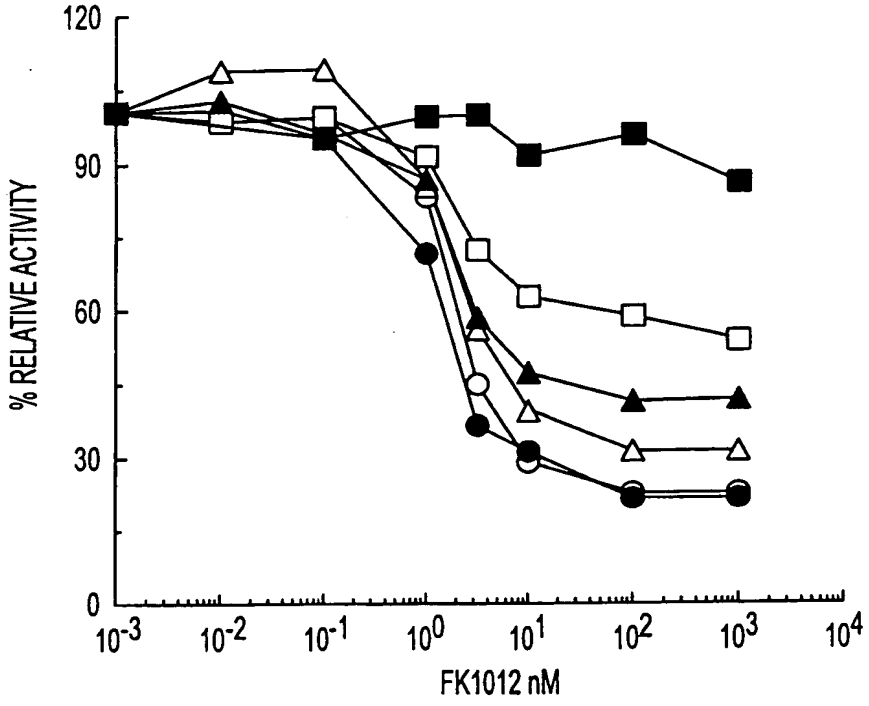
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
FK1012 nM

FIG. 5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90
60
30
0
% RELATIVE ACTIVITY
10^{-3}
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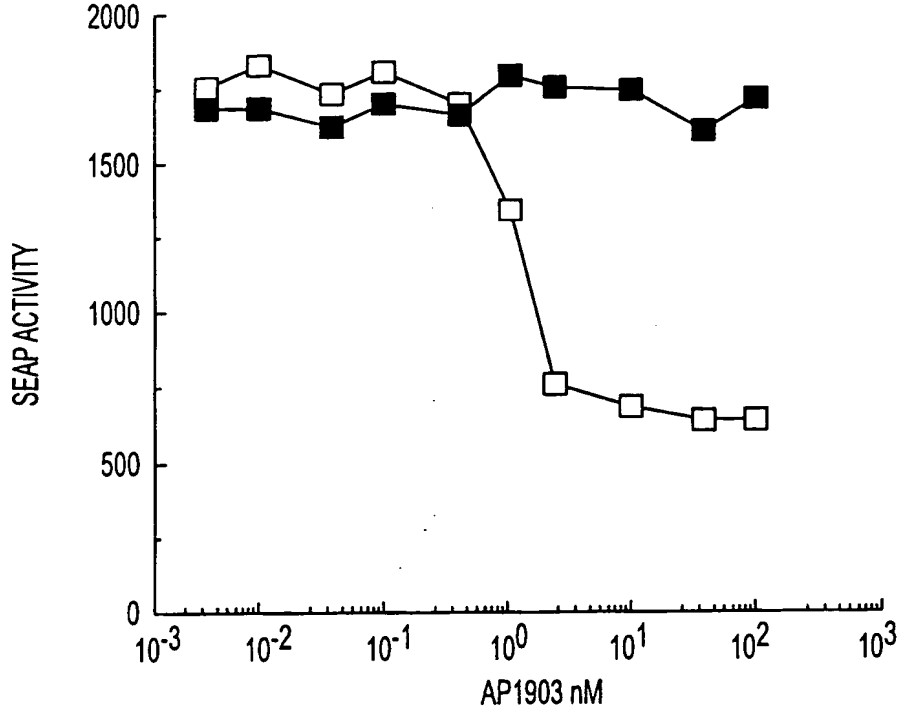
10^{3}
10^{4}
FK1012 nM

FIG. 6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500
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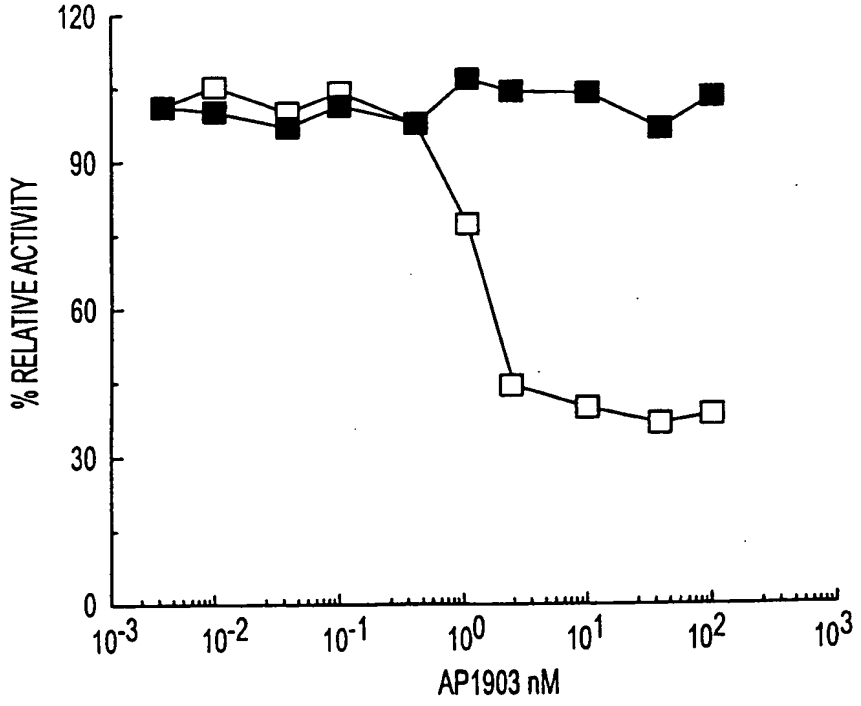
10^{3}
AP1903 nM

FIG. 7


120
90
60
30
0
% RELATIVE ACTIVITY
10^{-3}
10^{-2}
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10^{0}
10^{1}
10^{2}
10^{3}
AP1903 nM

FIG. 8

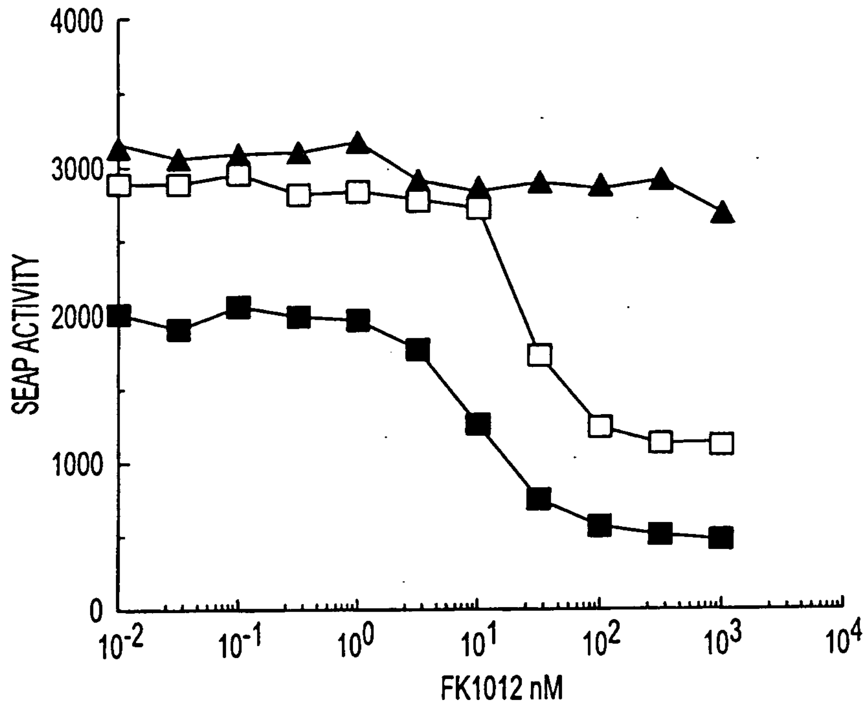


FIG. 9

F_V2 - CASP - 3						F_V2 - CASP 3/S163					
HOURS						HOURS					
8	4	2	1	.5	0	8	4	2	1	.5	0

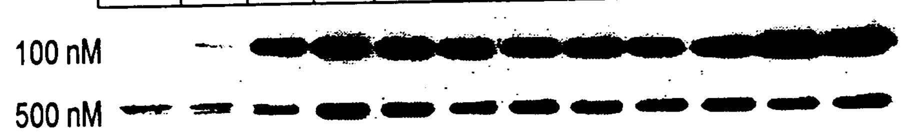


FIG. 10

F_V2 - CASP - 3						F_V2 - CASP 3/S163					
AP1903 nM						AP1903 nM					
100	32	10	3.2	1	0	100	32	10	3.2	1	0

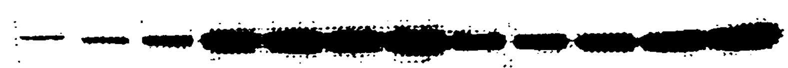


FIG. 11

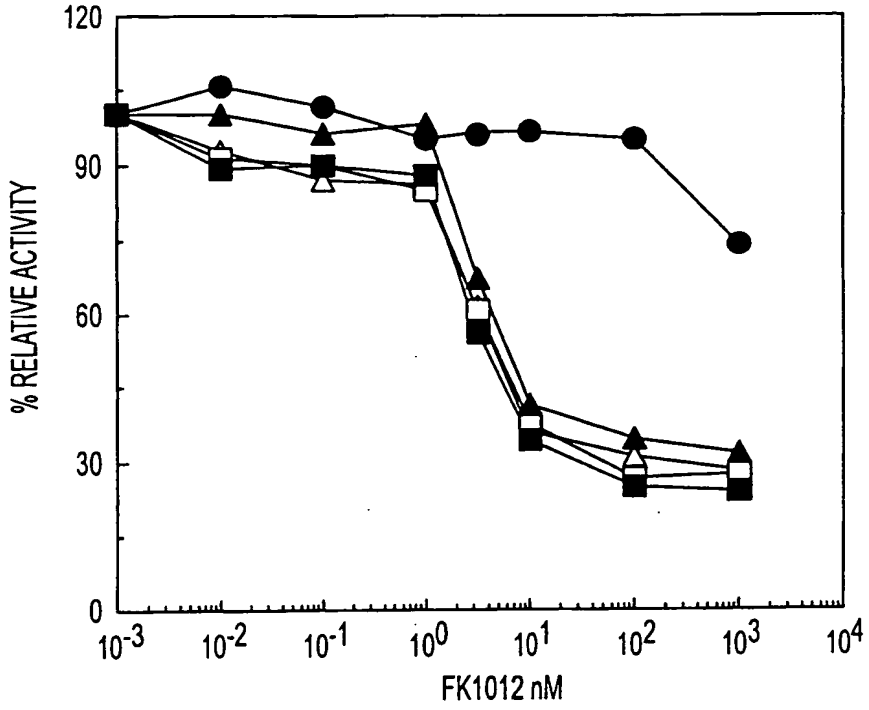


FIG. 12

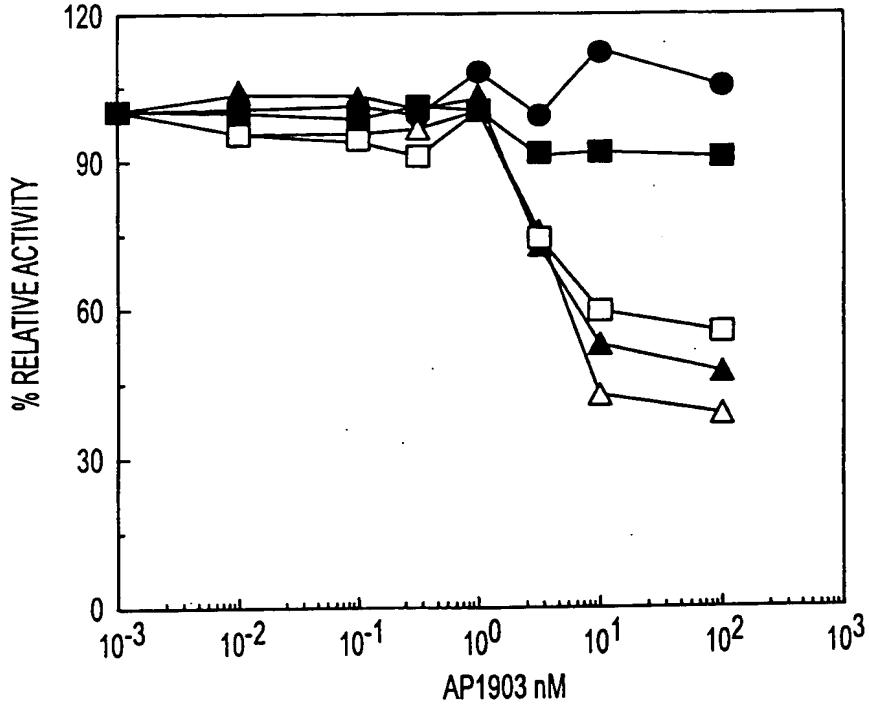


FIG. 13

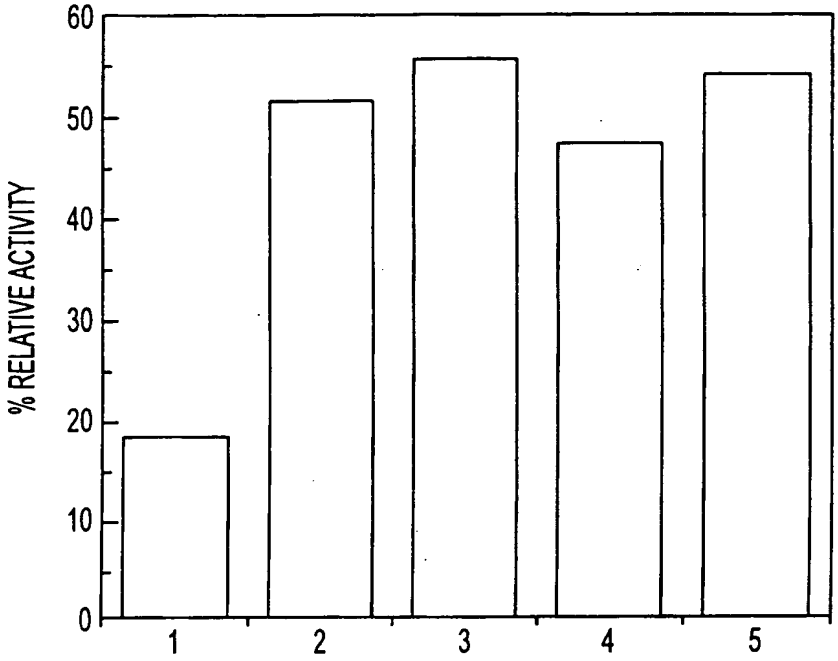

% RELATIVE ACTIVITY
60
50
40
30
20
10
0
1
2
3
4
5

FIG. 14

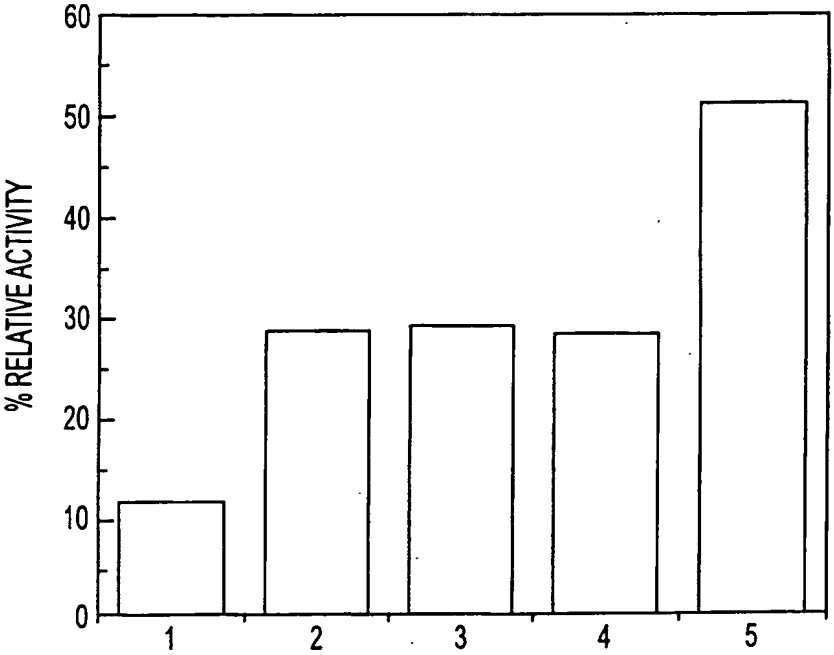

% RELATIVE ACTIVITY
60
50
40
30
20
10
0
1
2
3
4
5

FIG. 15

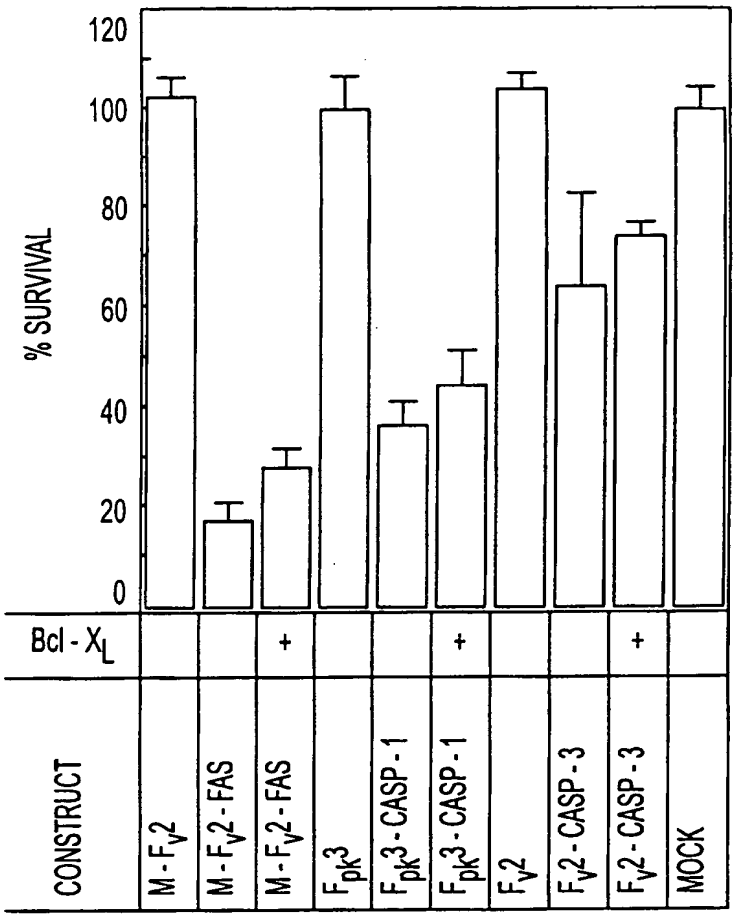

120
100
80
60
40
20
0
% SURVIVAL
Bcl - XL
+
+
+
CONSTRUCT
M - Fv2
M - Fv2 - FAS
M - Fv2 - FAS
Fpk3
Fpk3 - CASP - 1
Fpk3 - CASP - 1
Fv2
Fv2 - CASP - 3
Fv2 - CASP - 3
MOCK

FIG. 16

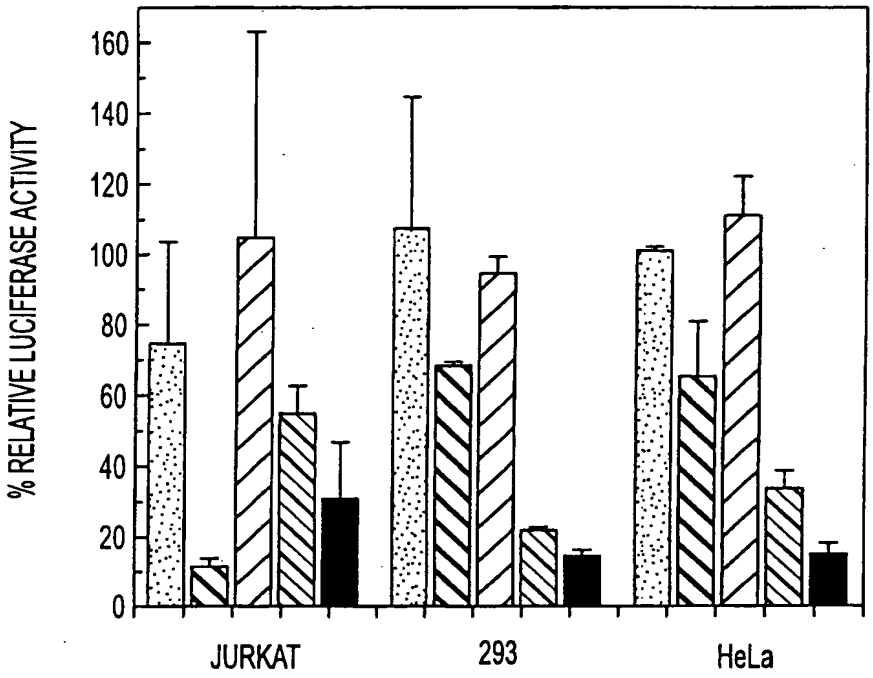

% RELATIVE LUCIFERASE ACTIVITY
160
140
120
100
80
60
40
20
0
JURKAT
293
HeLa

FIG. 17

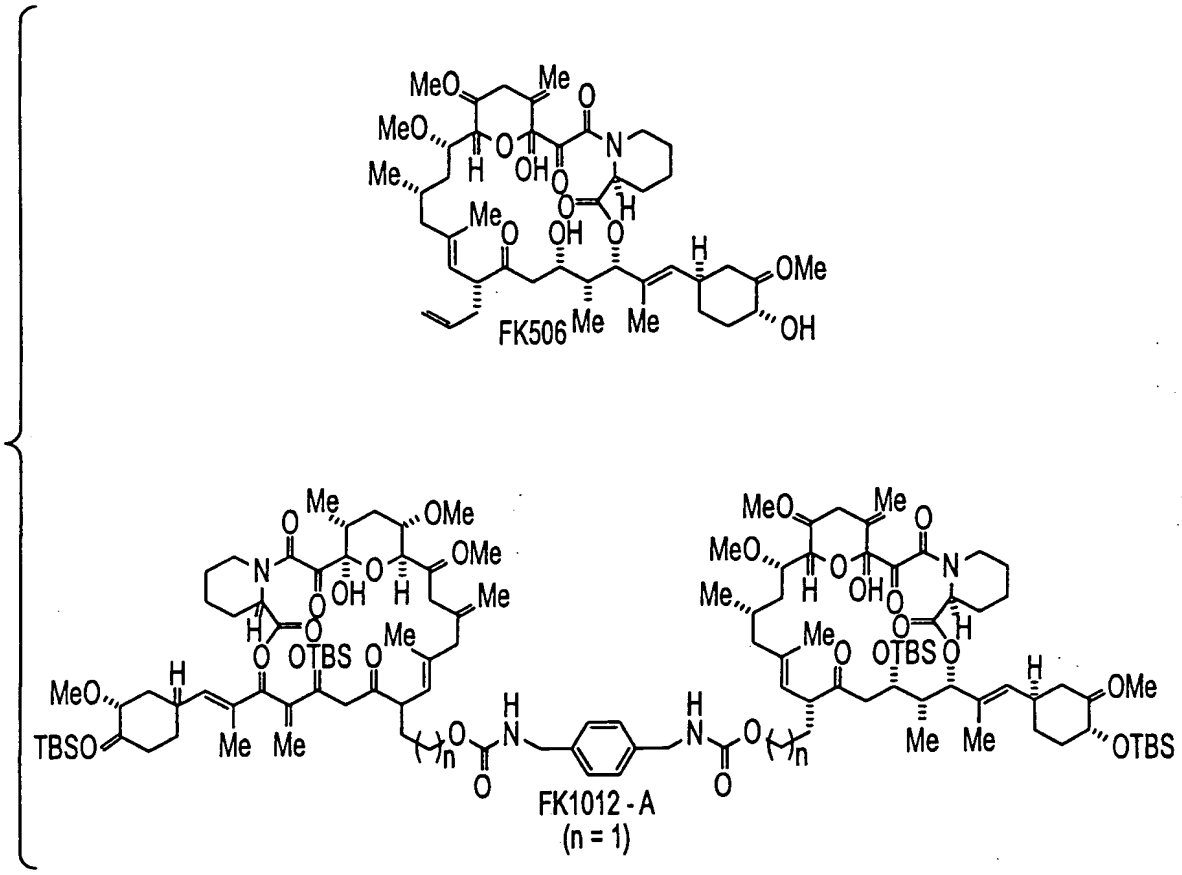


FIG. 18

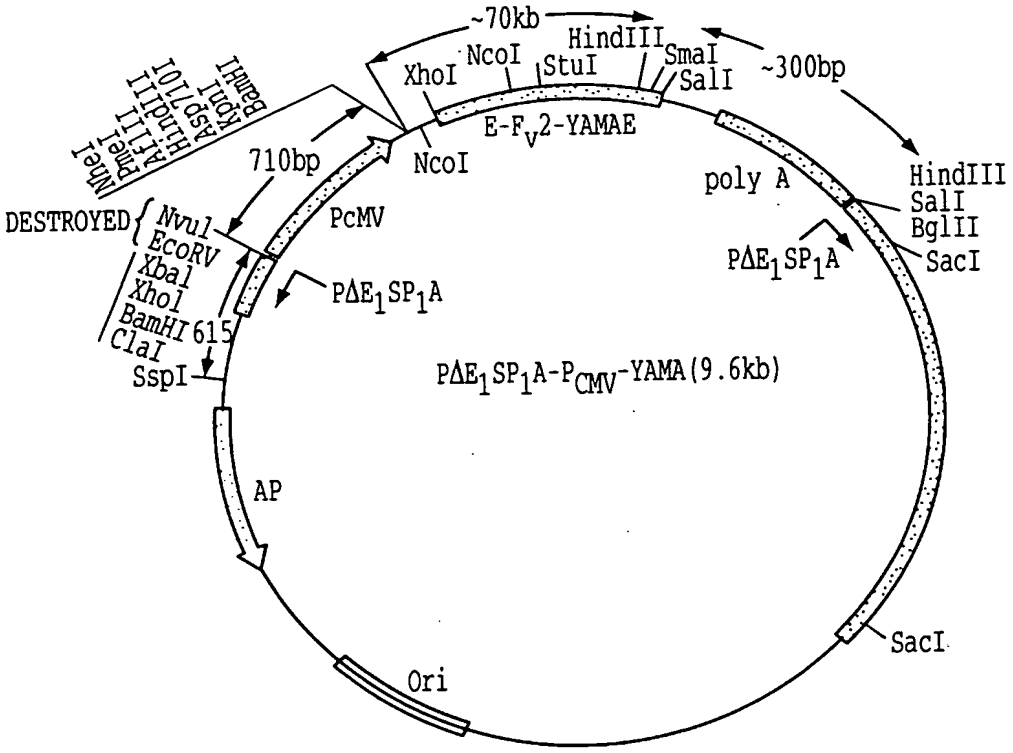


FIG. 19A

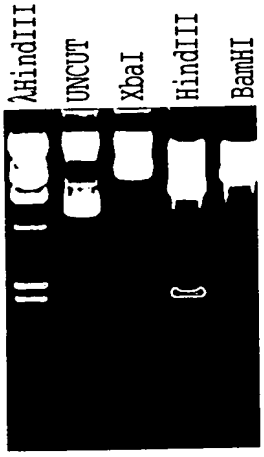


FIG. 19B

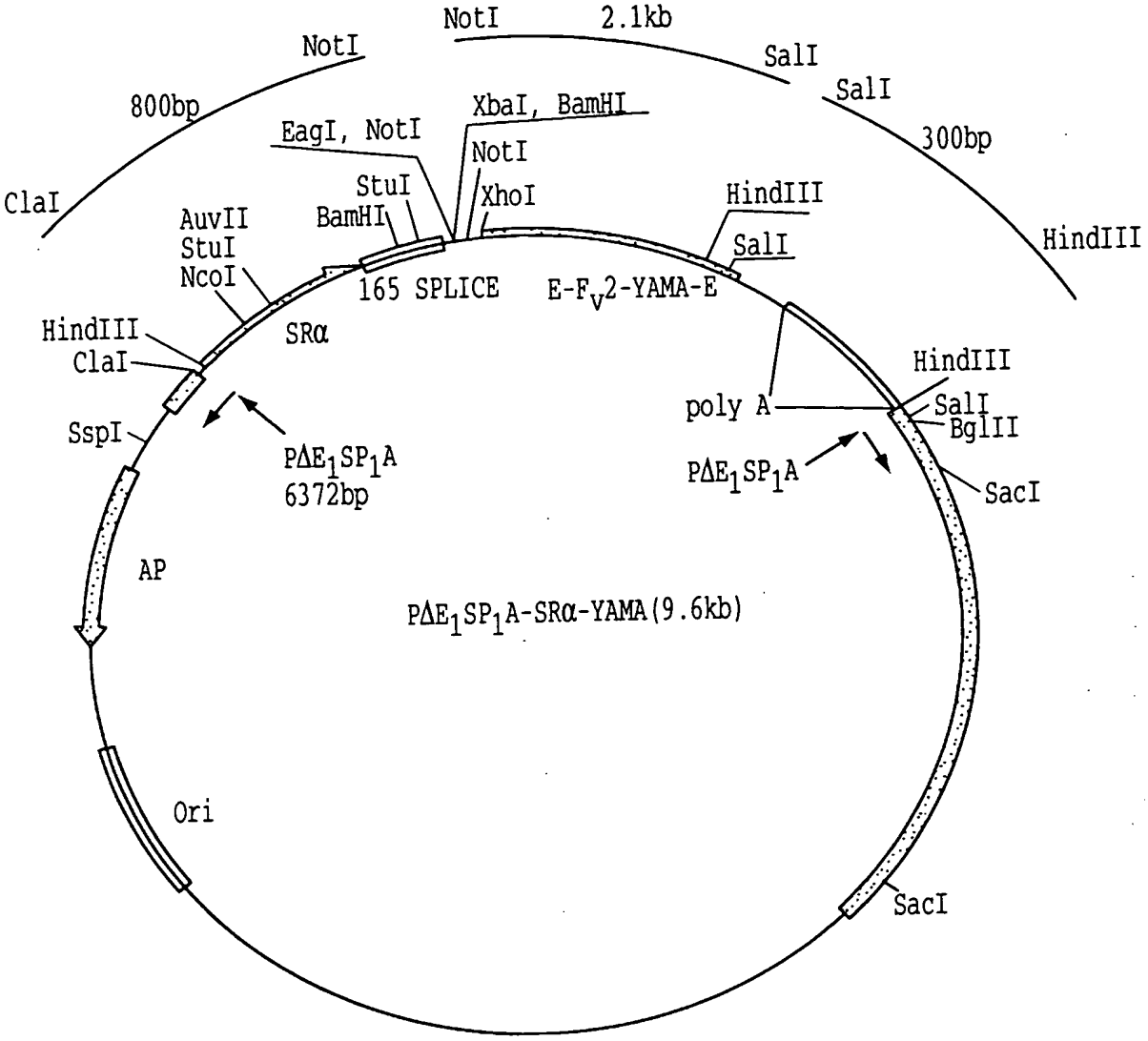


FIG. 20A

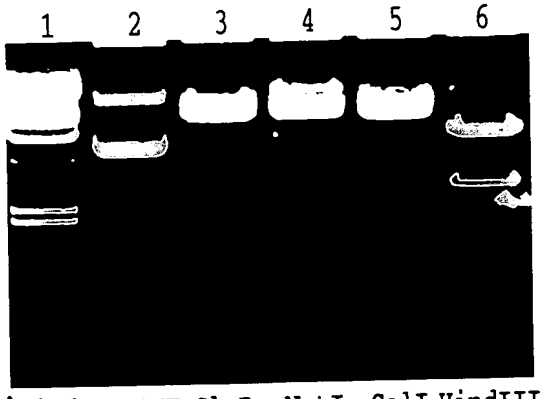


FIG. 20B

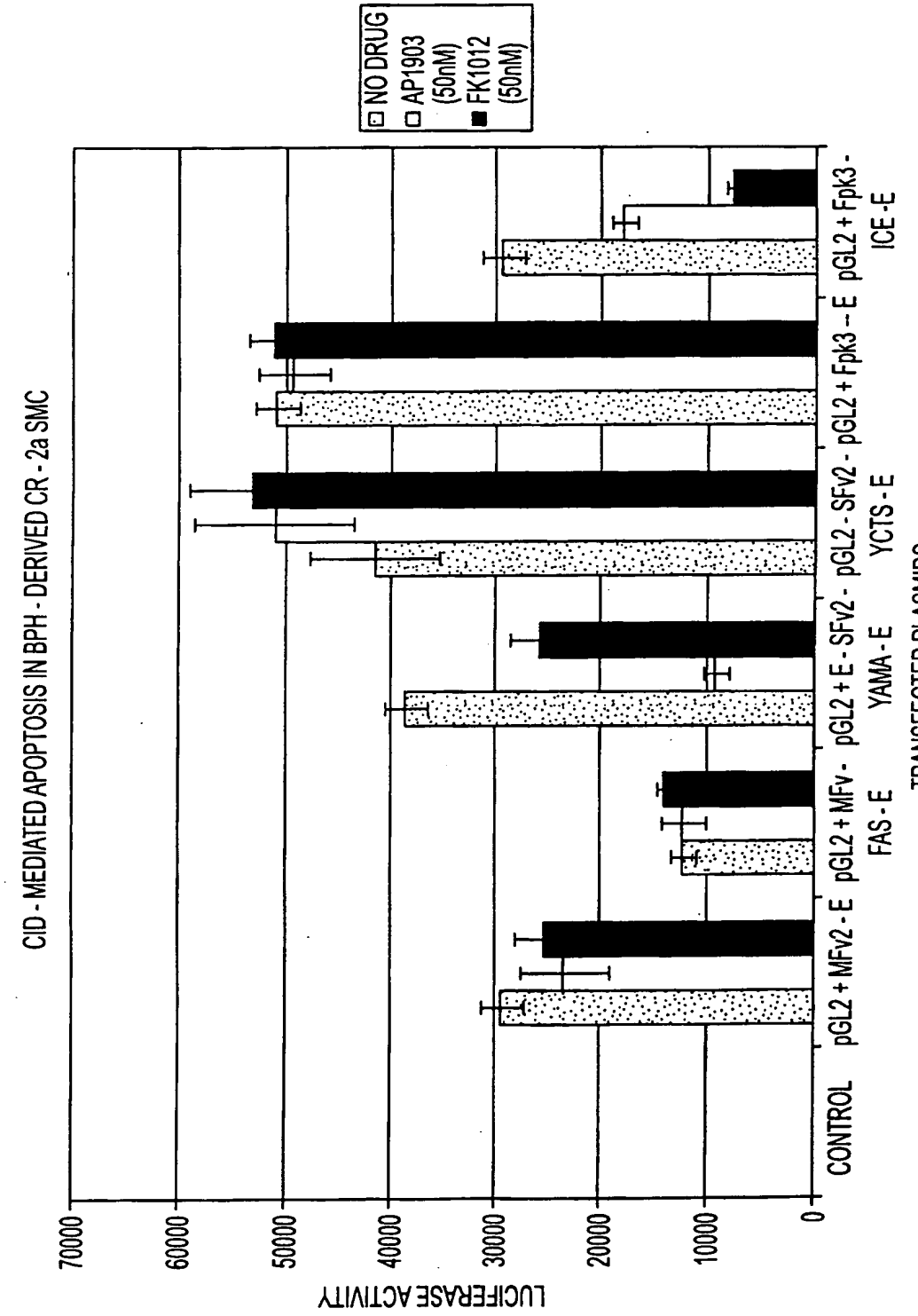


FIG. 21

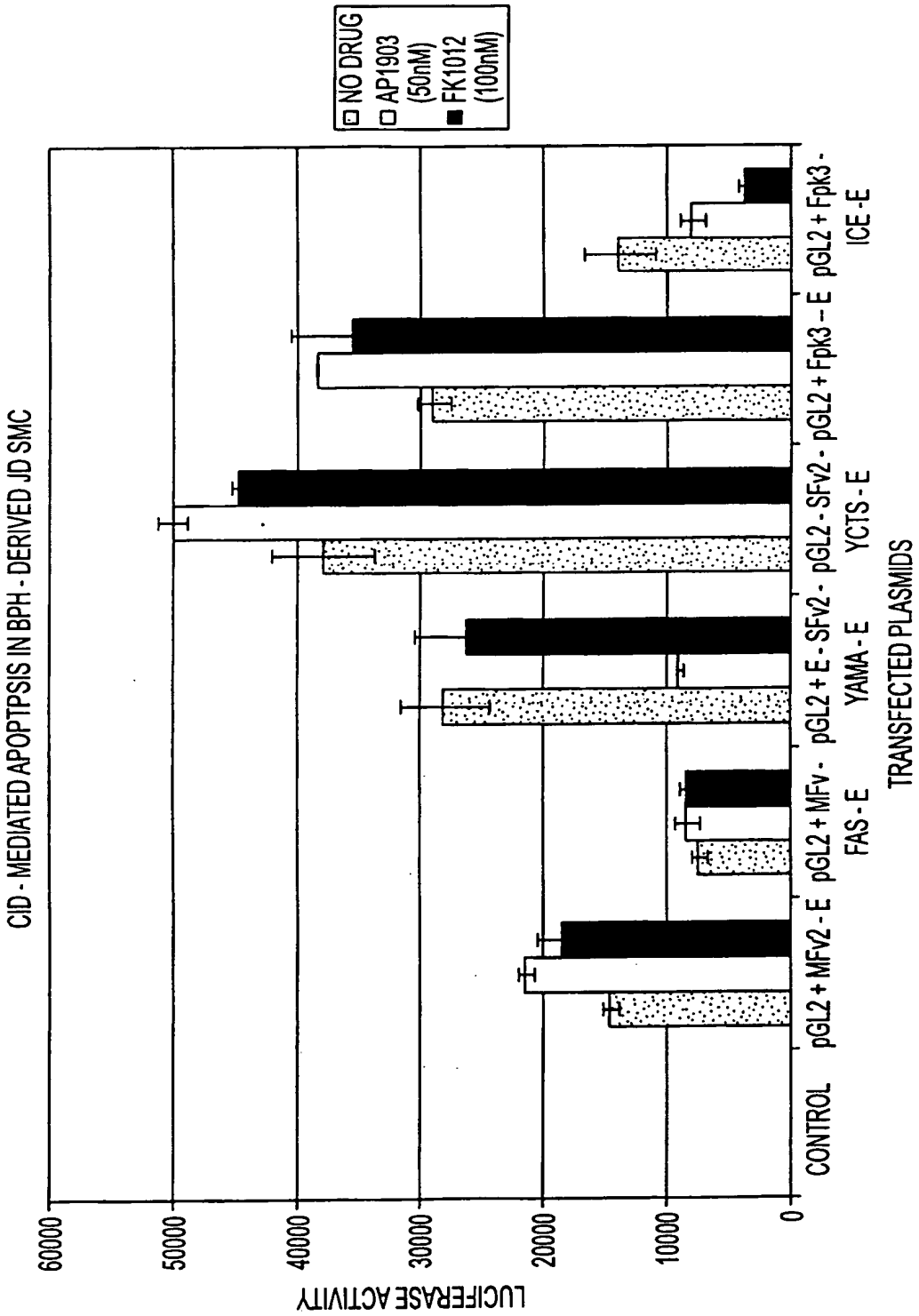

NO DRUG
AP1903 (50nM)
FK1012 (100nM)
CID - MEDIATED APOPTPSIS IN BPH - DERIVED JD SMC
LUCIFERASE ACTIVITY
60000
50000
40000
30000
20000
10000
0
CONTROL
pGL2 + MFv2 - E
pGL2 + MFv - FAS - E
pGL2 + E - SFv2 - YAMA - E
pGL2 - SFv2 - YCTS - E
pGL2 + FpK3 – E
pGL2 + FpK3 - ICE - E
TRANSFECTED PLASMIDS

FIG. 22

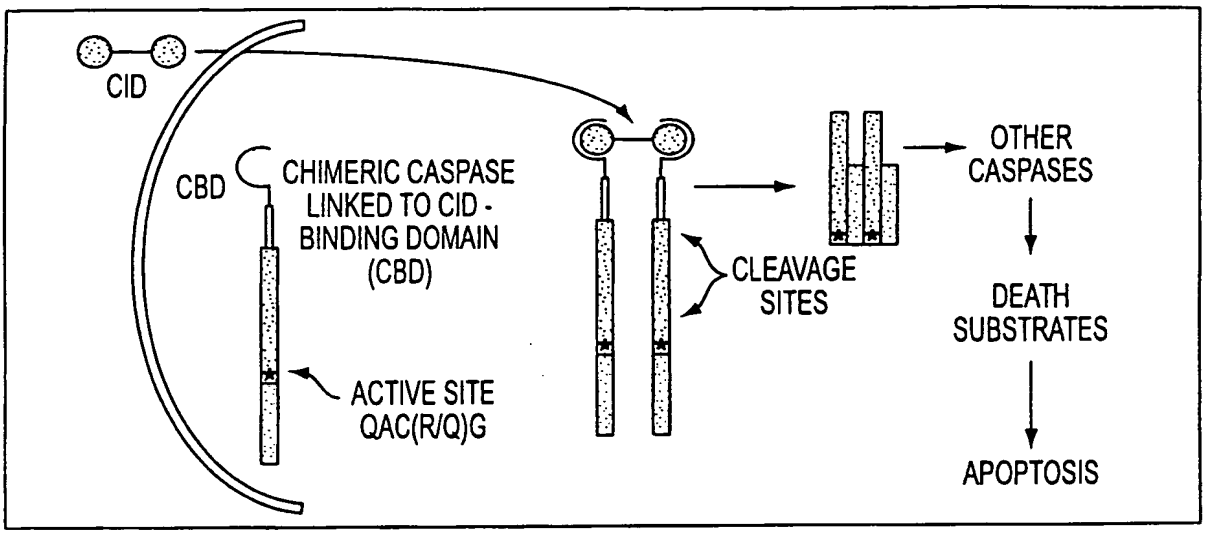


FIG. 23A

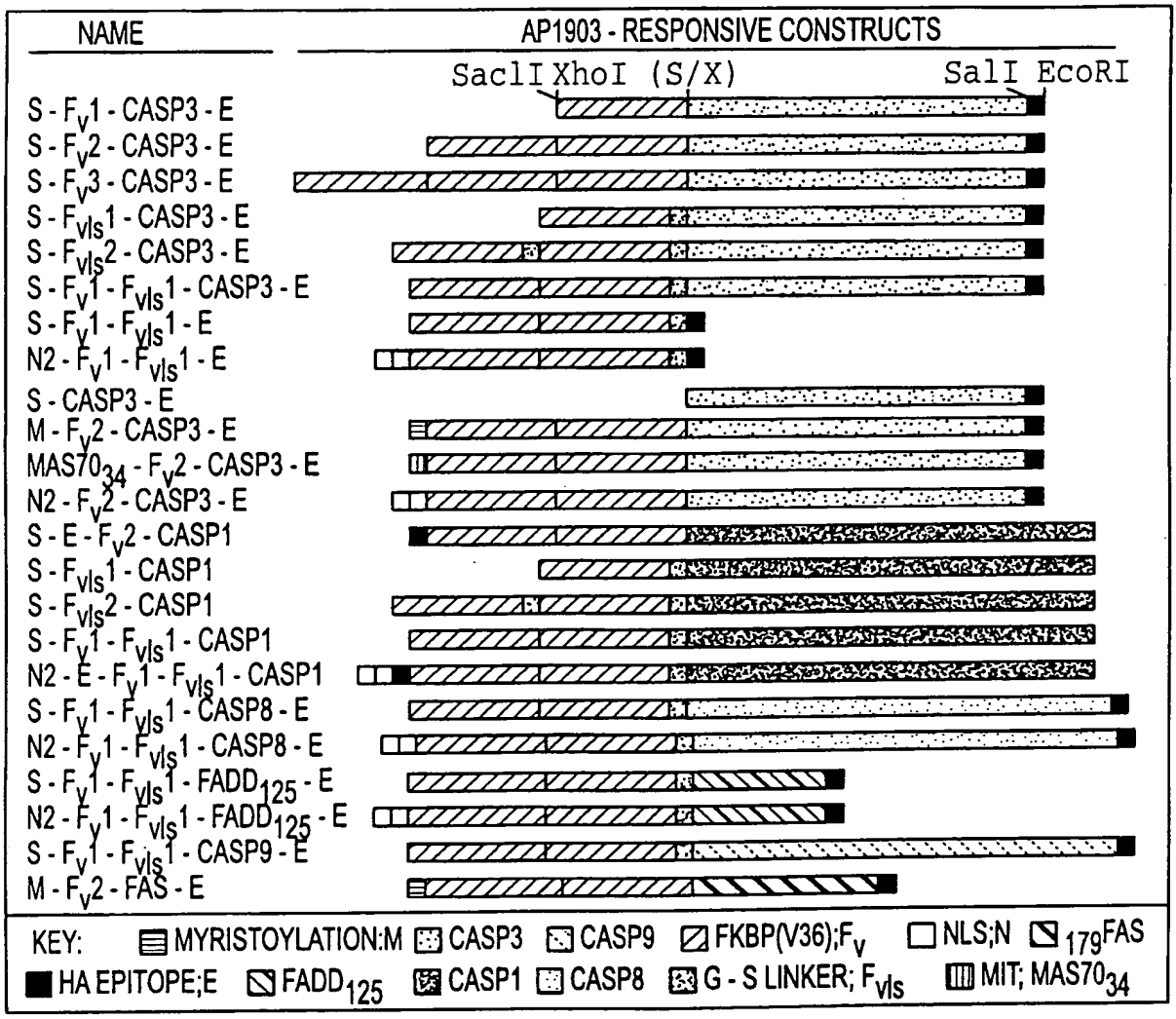


FIG. 23B

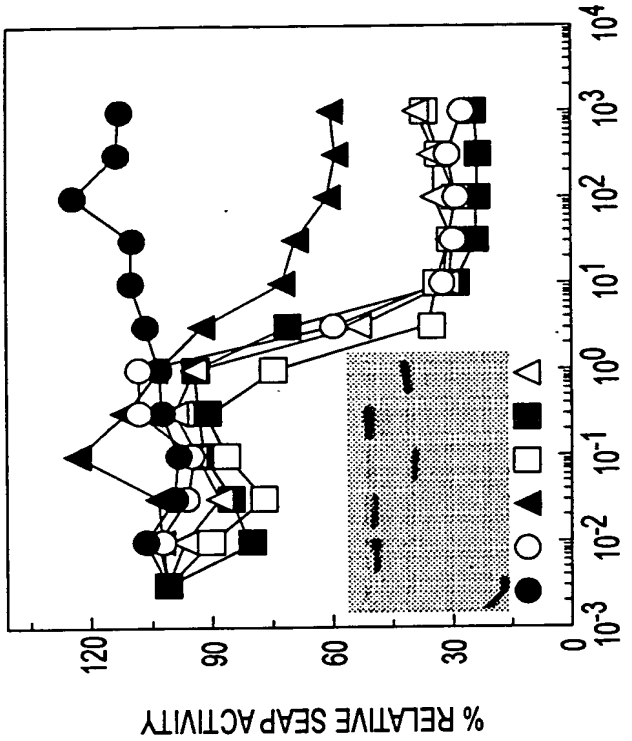


FIG. 24B

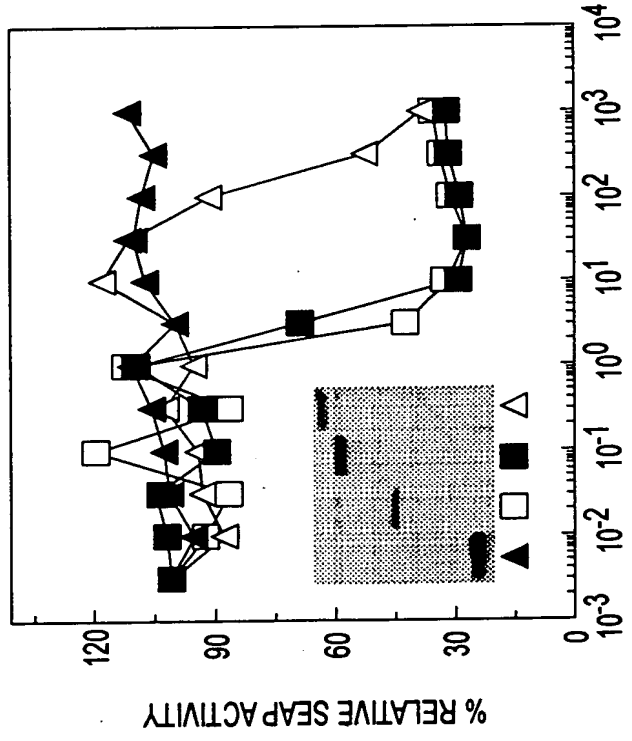


FIG. 24A

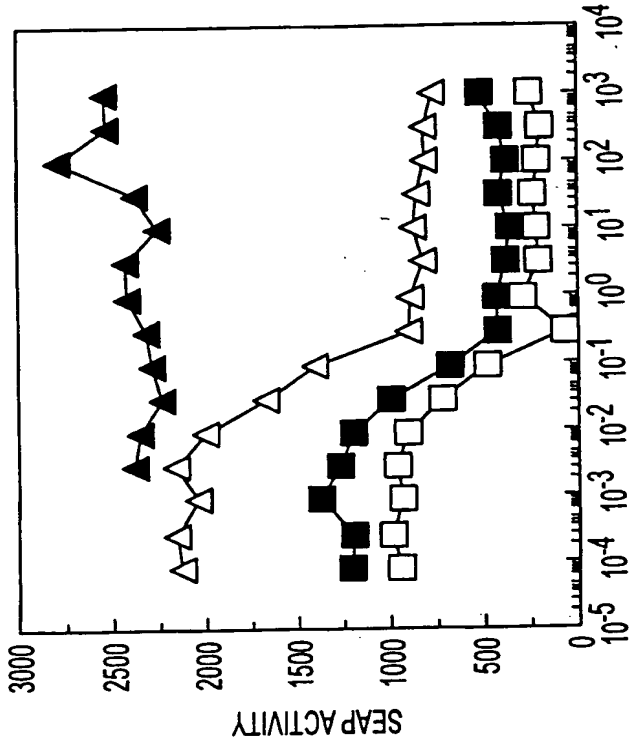


FIG. 24D

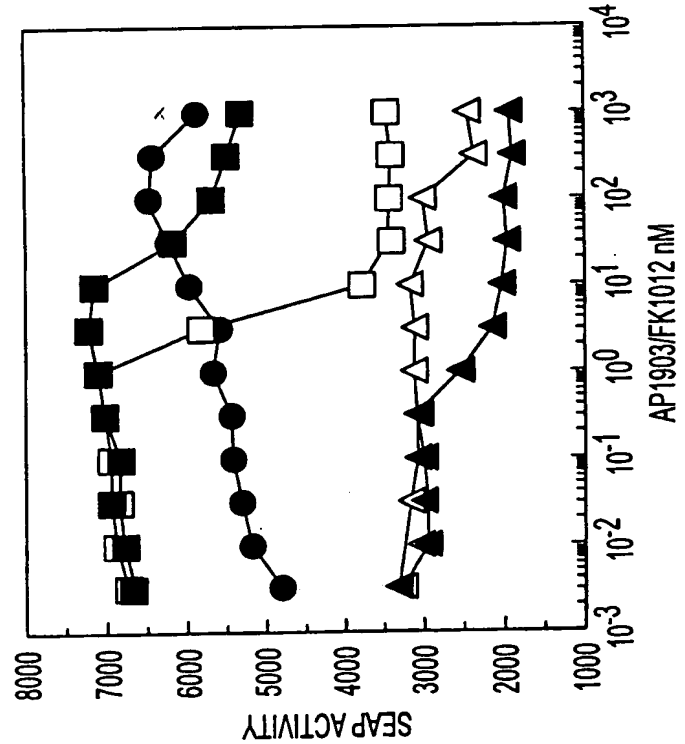


FIG. 24C

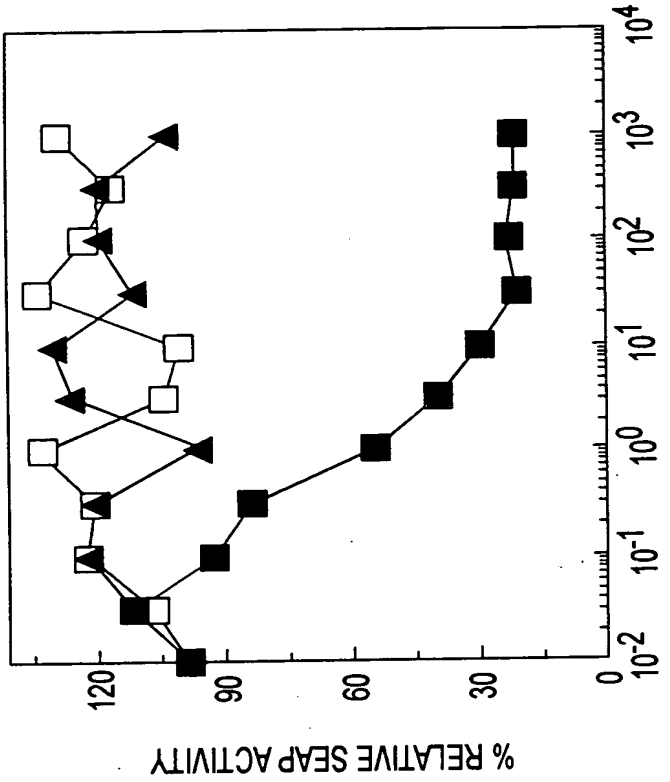


FIG. 25B

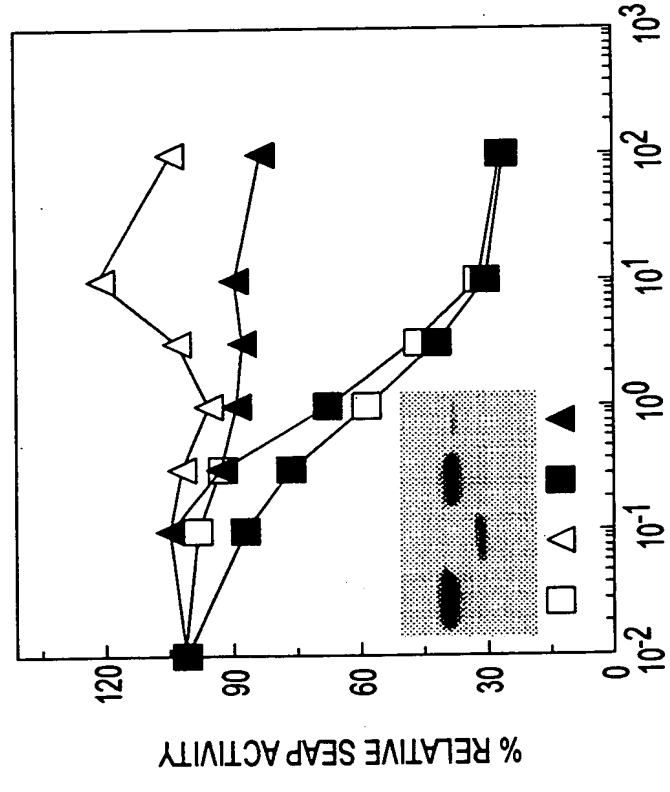


FIG. 25A

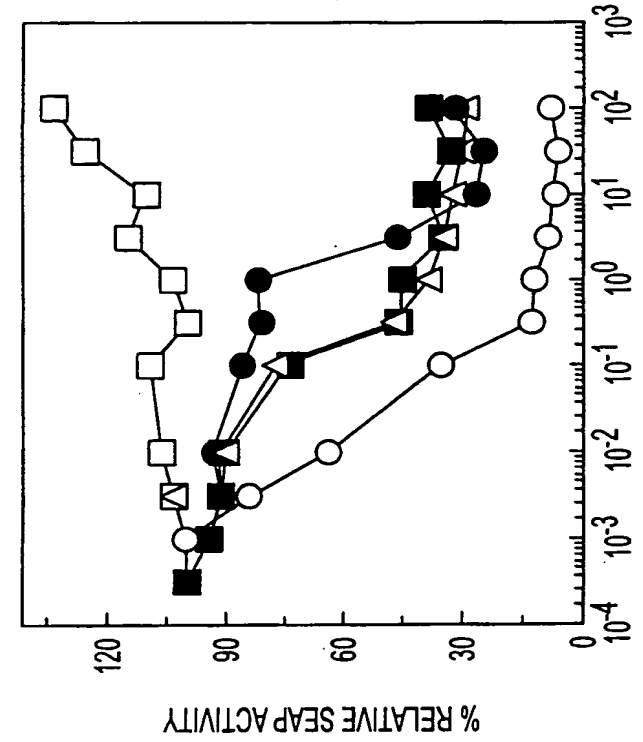


FIG. 25D

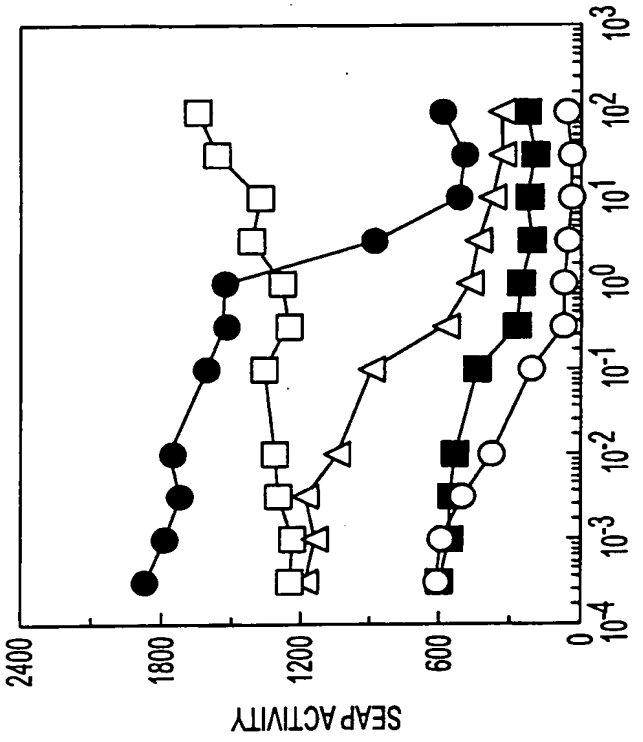


FIG. 25C

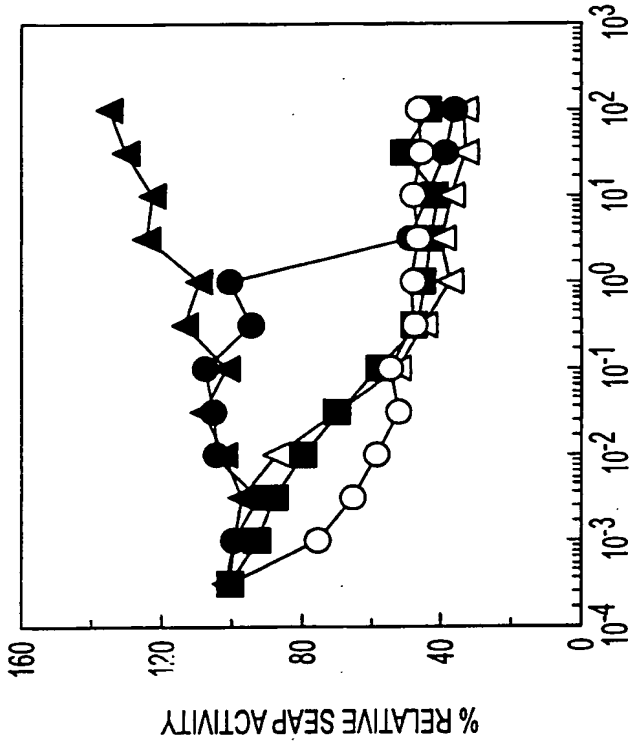


FIG. 25F

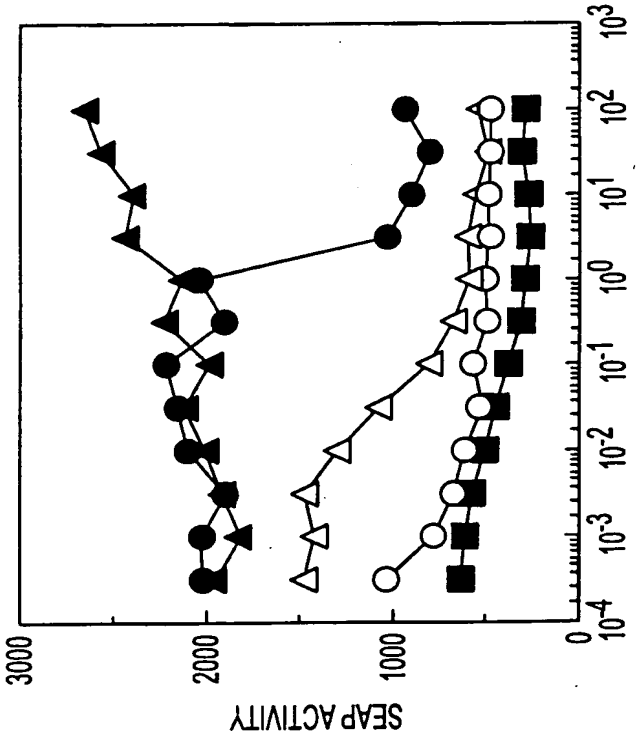


FIG. 25E

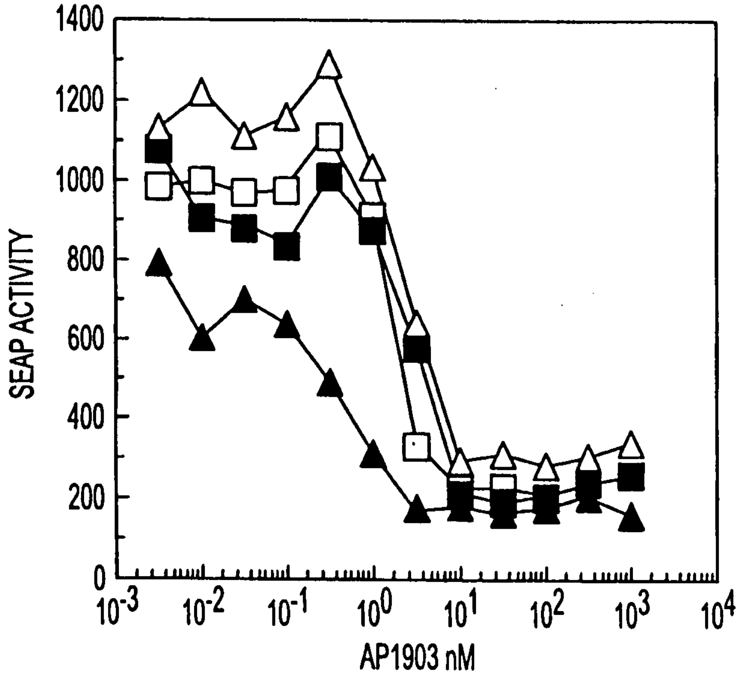


FIG. 26A

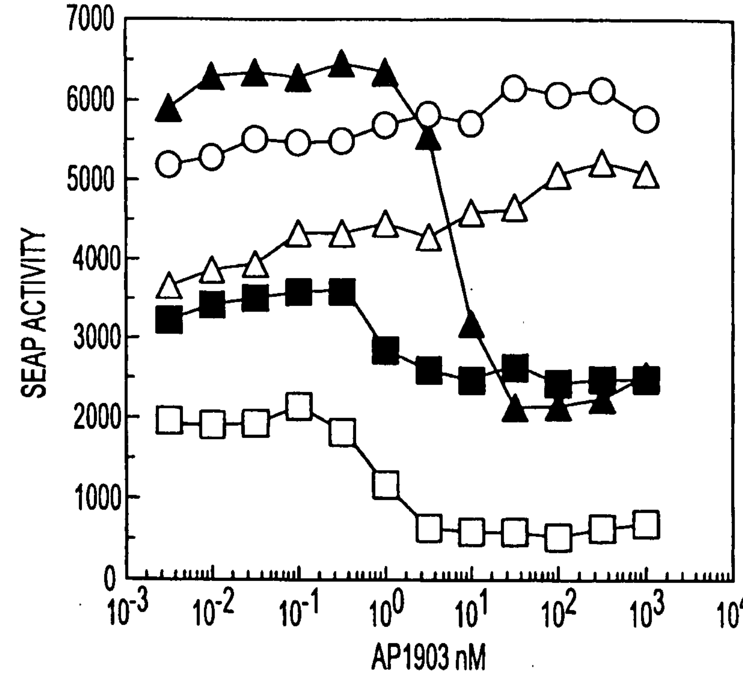


FIG. 26B

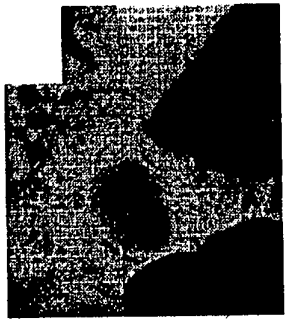


FIG. 26C

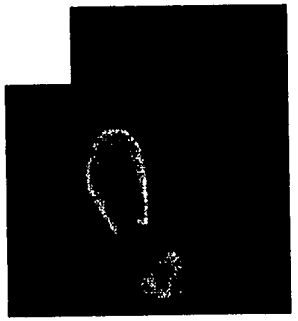


FIG. 26D

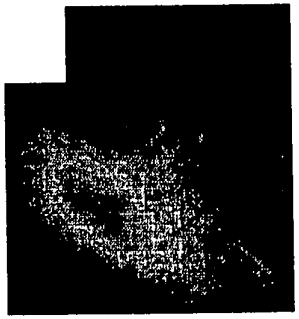


FIG. 26E

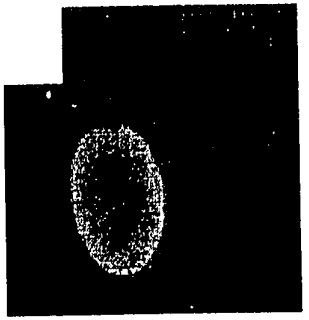


FIG. 26F

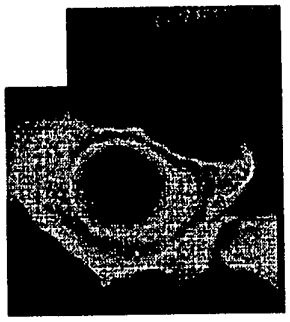


FIG. 26G

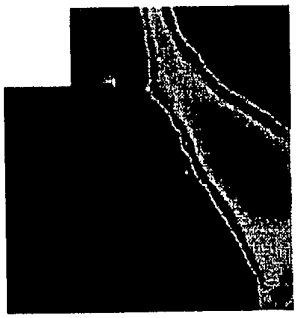


FIG. 26H

FIG. 26I

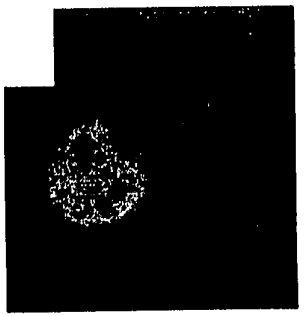


FIG. 26J

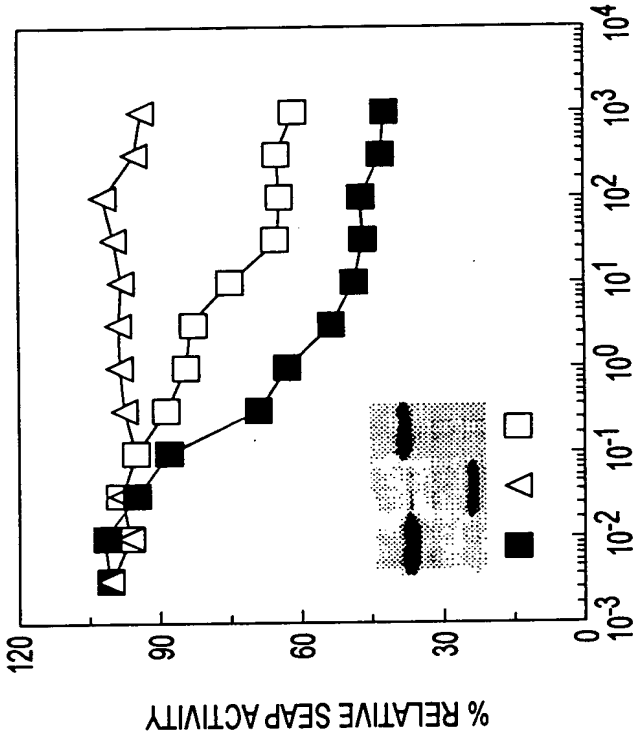


FIG. 27C

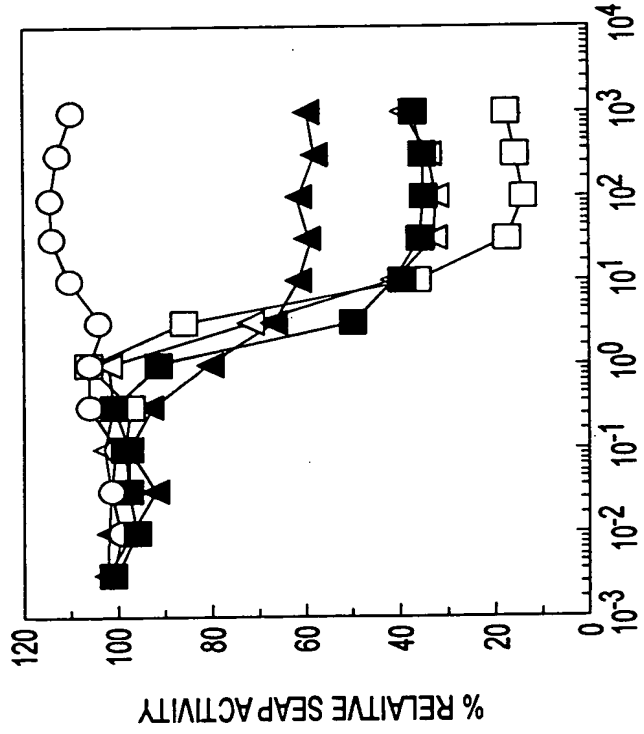


FIG. 27D

Generation of pAd Track-CMV~F_{vls}1-YM-E
And pAdTrack-CMV ~E-F_v1-F_{vls}1-ICE st

1. Cut pAdTrack-CMV-with EcoRV+Not. I

   1μg/2μl (pAd Track-CMV) + 1μl buffer H
   + 1μl EcoRV + 1μl Not I +H_2O 5μl
   37°C, 2 hr

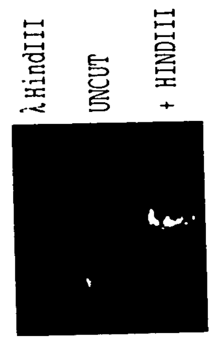


Figure 28A

2. Cut pSH1/S-F_{vls}1-YM-E with Not I+EcoRI (got 2.2Kb F_{vls}1-YM-E)
   2μg/1.2μl (pSH$_1$/S$_2$ F_{vls}1-YM-E+ 2μl EcoRI
   +2μl Buffer H. + H_2O 14.8 μl. 37°C, 1 hr.

   or

   E-F_v1-F_{vls}1-ICEst
   2μg/3.2μl
   12.8 H_2O

   Blunt
   + 10μM dNTP 12μl (10μM/each)+2μl Buffer B
   14μl H_2O + 4U/2μl Klenow, 25°C, 30min;+EDTA→10 min.
   Inactivate Klenow by heating at 75°C, 10 min. phenol: chloroform extraction

   then + 2μl Not I. 37°C, 1hr.

3. Run gel, cut bands, pool together, Gene Clean. Elute in 40μl

4. Ligation

   Elutent 16μl + 2μl 10X ligation buffer
   + 2μl T4 ligase, 16°C, overnight

4. Transformation

5. Miniprep with Qiagen Kit

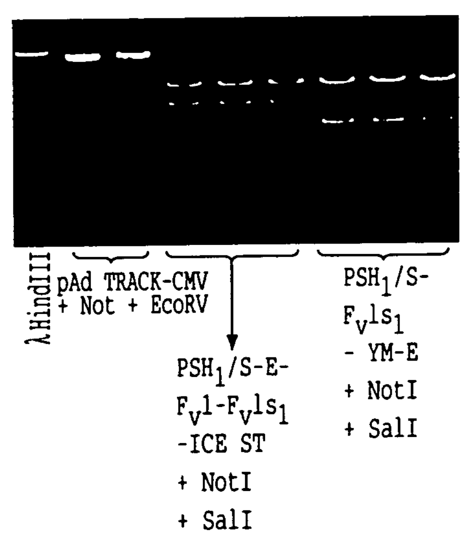


Figure 28B

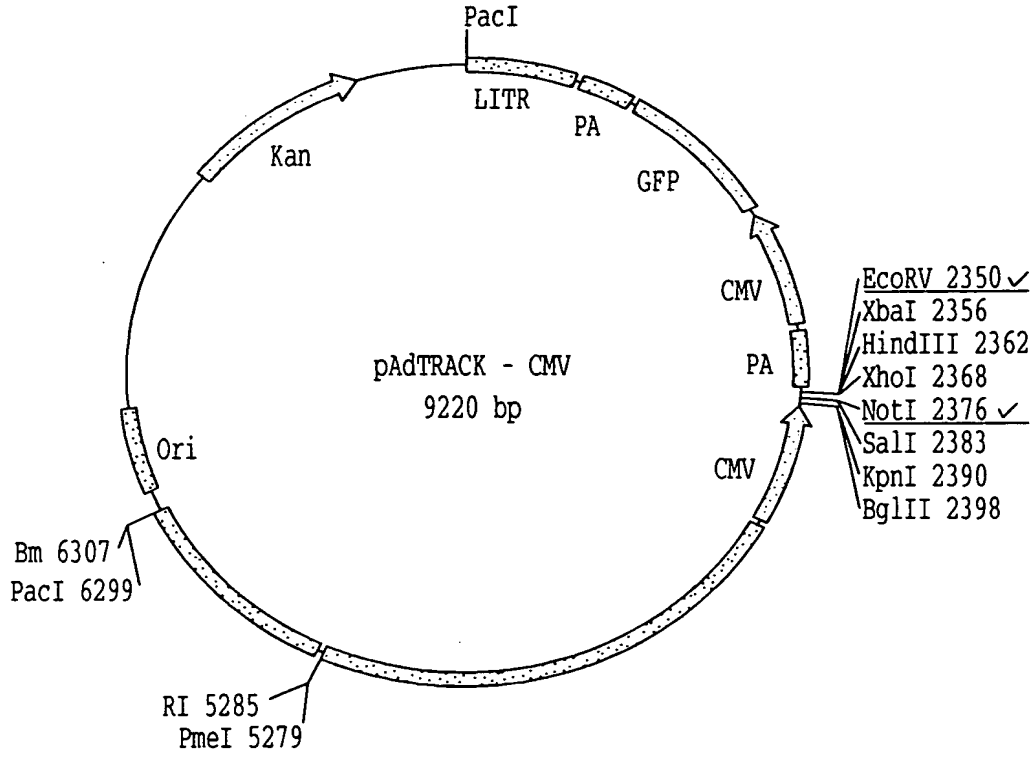


FIG. 29A

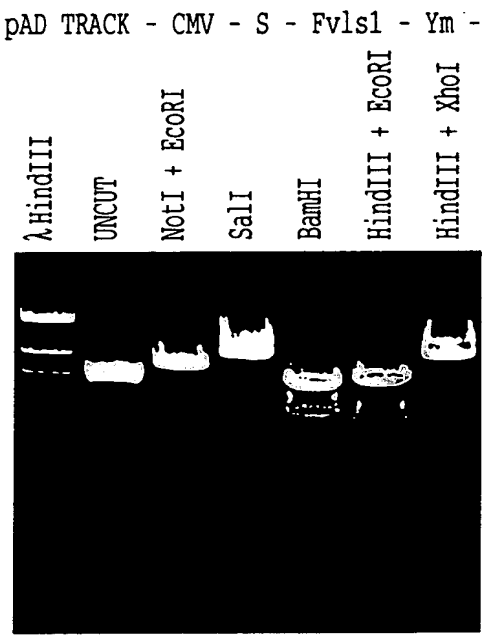


FIG. 29B

FIG. 29C

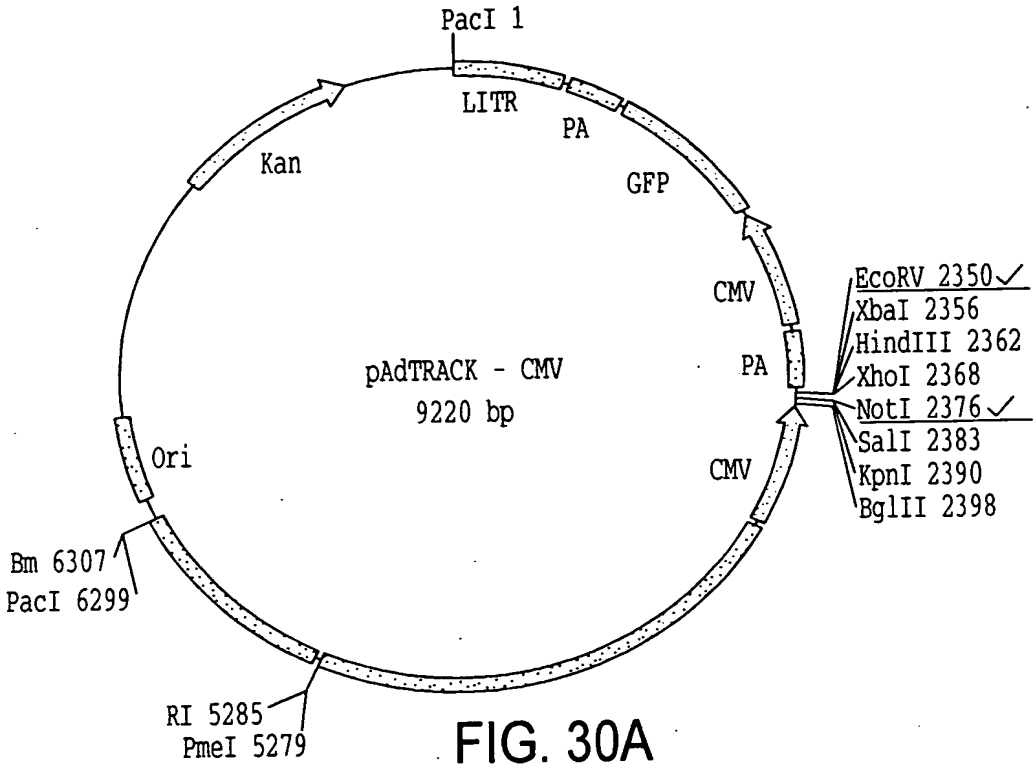


FIG. 30A

pAD TRACK - CMV - S - Fv1 - Fvls1 - ICEst

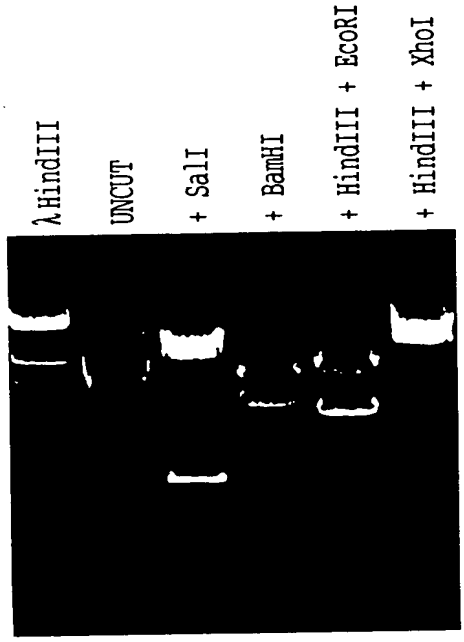


FIG. 30B

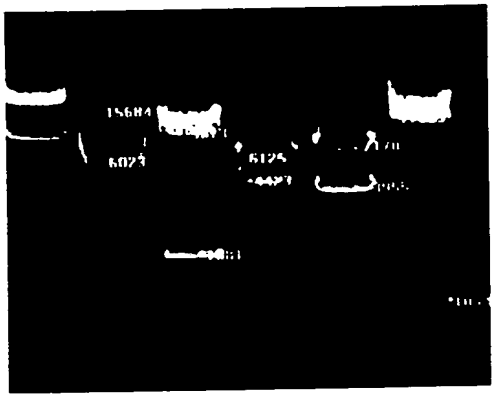


FIG. 30C

FIG. 30D

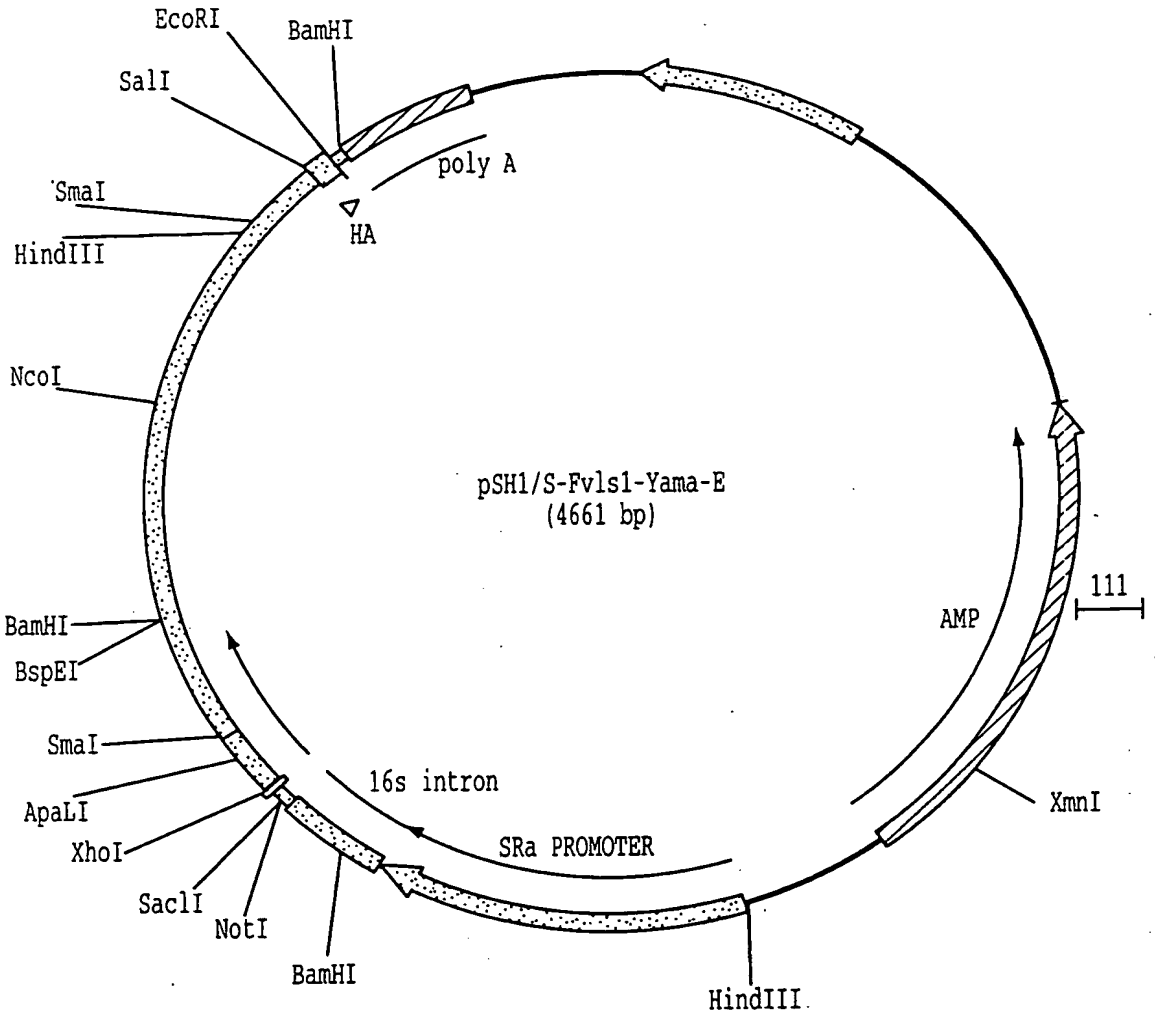

EcoRI
BamHI
SalI
SmaI
HindIII
NcoI
BamHI
BspEI
SmaI
ApaLI
XhoI
SacII
NotI
BamHI
poly A
HA
pSH1/S-Fvls1-Yama-E
(4661 bp)
16s intron
SRa PROMOTER
AMP
111
XmnI
HindIII

FIG. 32

Generation of Ad-GFP-CMV-YM-E
Ad-GFP-CMV-E-ICE

1. Co-transform E. coli BJ SH3 with pAdEasy -1 and pAdTrack-CMV-$F_{vls}1$-YM-E or pAd Track-CMV-E-F_v1-$F_{vls}1$-ICE st.

a. Linearize the shuttle plamids with PmeI
1μg of pAdTrack-CMV-$F_{vls}1$-YM-E
or 1μg of pAdTrack-CMV-E-F_v1-$F_{vls}1$-ICEst
+ 1μl Buffer + $H_2O \rightarrow 9\mu$l, + 1μl PmeI
37°C, 2hr
phenol-chloroform extraction, ethanol precipitation and resuspend in 6μl H_2O

b. Co-transformation: with 100ng pAdEasy -1
20μl of BJ5183
2.0mm cuvettes
at 2,500V, 200 Ohms, 25 μFD

c. Selection:
pick 10 smallest colonies, miniprep. check with PacI.
Retransform-XL-1 Blue with the correct plasmid, miniprep, recheck with Pac I. Midiprep.

d. Transfect 293 cells with FuGene.

90% confluence 293 cells in G-well-plate
4μg plasmid/6μl FuGene/well

Figure 33

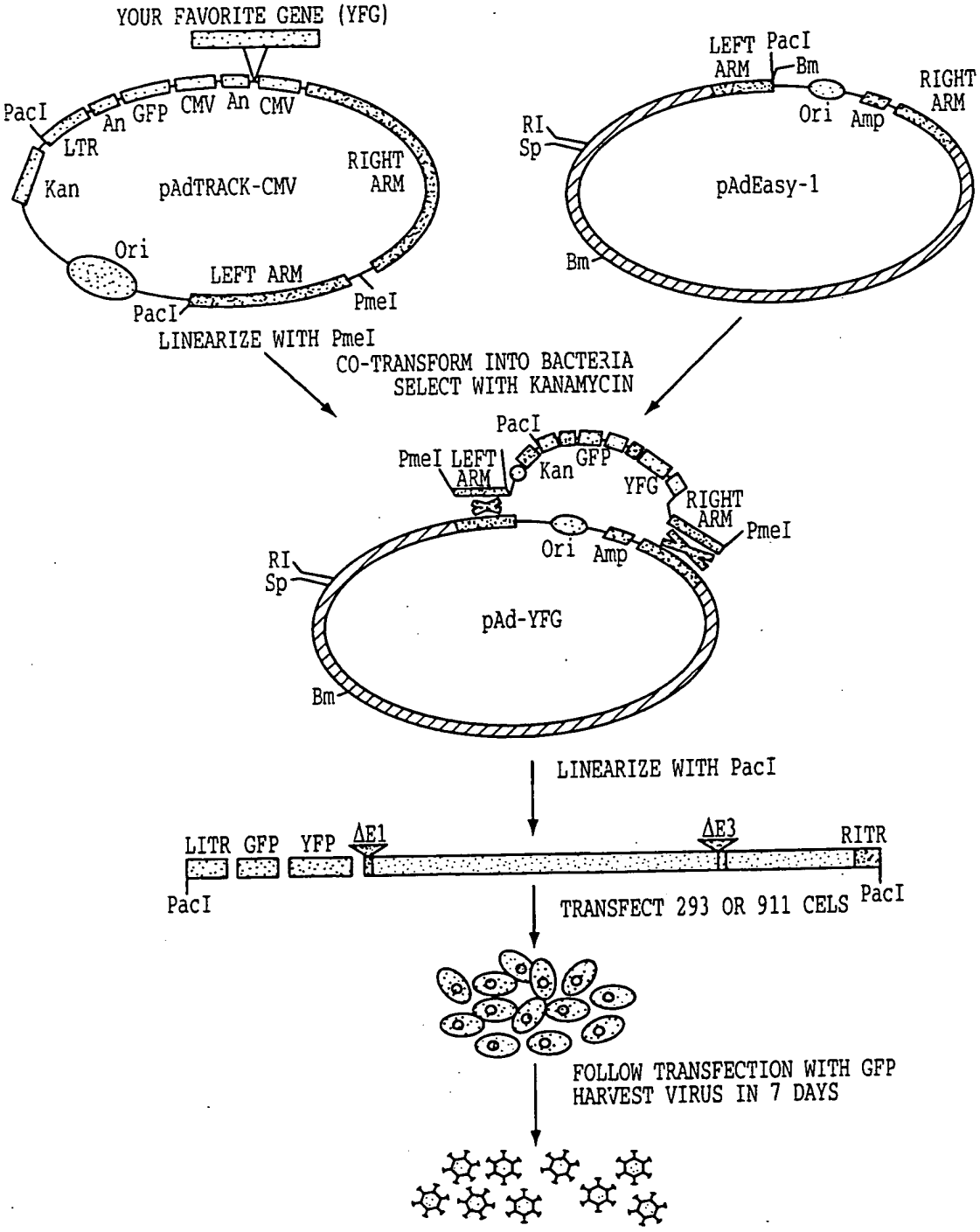
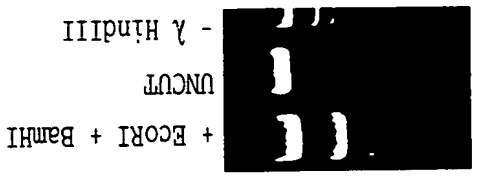

YOUR FAVORITE GENE (YFG)
PacI
An
GFP
CMV
An
CMV
LTR
Kan
pAdTRACK-CMV
RIGHT ARM
Ori
LEFT ARM
PacI
PmeI
LINEARIZE WITH PmeI
LEFT ARM
PacI
Bm
Ori
Amp
RIGHT ARM
RI
Sp
pAdEasy-1
Bm
CO-TRANSFORM INTO BACTERIA
SELECT WITH KANAMYCIN
PacI
PmeI
LEFT ARM
Kan
GFP
YFG
RIGHT ARM
PmeI
Ori
Amp
RI
Sp
pAd-YFG
Bm
LINEARIZE WITH PacI
LITR
GFP
YFP
ΔE1
ΔE3
RITR
PacI
PacI
TRANSFECT 293 OR 911 CELS
FOLLOW TRANSFECTION WITH GFP
HARVEST VIRUS IN 7 DAYS

FIG. 34



MINIPREP
082498

FIG. 35B

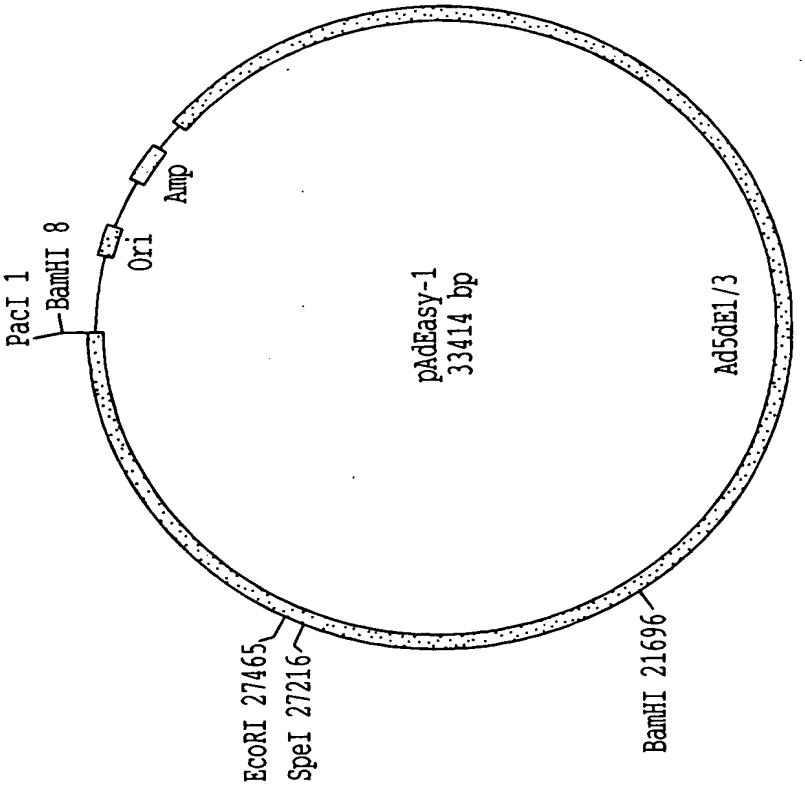


FIG. 35A

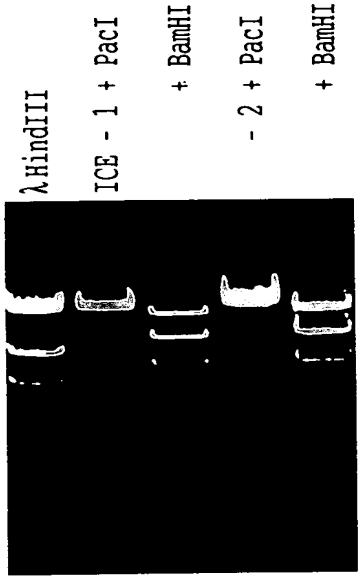


FIG. 36A

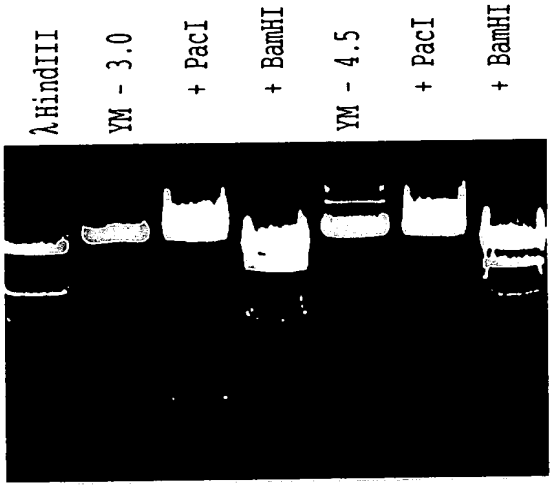


FIG. 36B

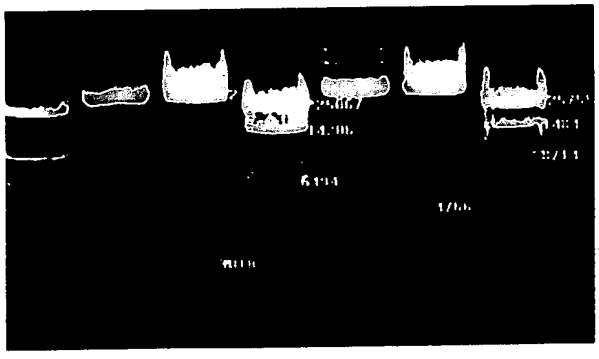


FIG. 36C

09/647418

Generation of Ad-CMV-E-ICE

Construction of pshuttle-CMV-F_v1-F_{vls}1-ICEst and Ad-CMV-E-ICE

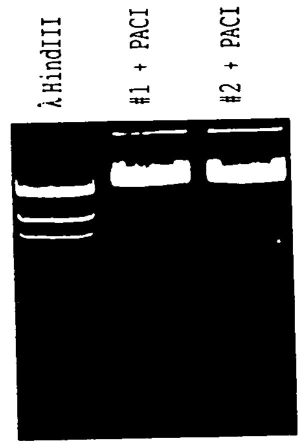


Figure 37A

1. Cut pshuttle-CMV with EcoRV+Not I
   1μg/2μl (pshuttle-CMV) + 1μl Buffer H
   + 1μl EcoRV + 1μl NotI + H_2O 5μl
   37°C, 2hr.

2. Cut pSH1/S-E-F_v1-F_{vls}1-ICEst with
   Not I + EcoRI (get E-F_v1-F_{vls}1-ICE st)

   3μg/4μl + 3μl Buffer H. + 3μl EcoRI
   +H_2O 21μl, 37°C, 1hr

   Blunt
   + 10μM dNTP 18μl (10μM/each) +3μl Buffer B
   + 3 μl H_2O + 7μl, T4 DNA polymerase, 25°C, 30 min
   +EDTA→ 10 min, 75°C, 10 min
   phenol: chloroform extraction (twice)
   ethanol precipitation → 24μl H_2O

   Then
   + 3μl Buffer H + 3μl Not I
   37°C, 2hr

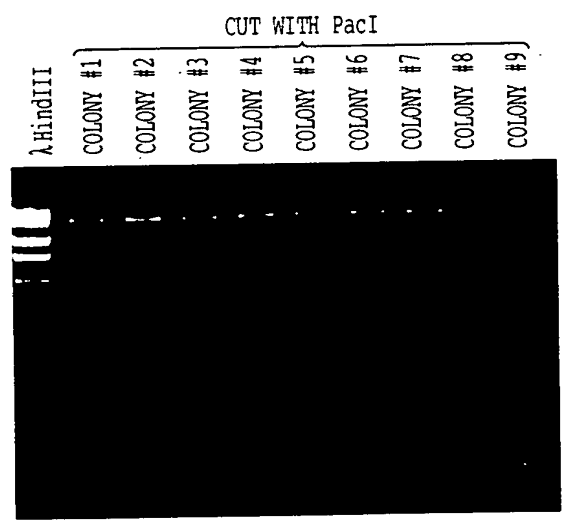


Figure 37B

3. Run Gel. Cut bands, pool together
   GeneClean, Elute in 40μl H_2O

4. Ligation
   Elutant 16μl+2μl 10X ligation Buffer
   +2μl T4 Ligase, 25°C, overnight

5. Transformation
6. Miniprep + Restriction Enzyme Cechking
7. Cut pshuttle-CMV-E-F_v1-F_{vls}1-ICEst with PmeI (1/5 of a miniprep), Cotransform E. coli BJ5183 with 100ng PAd Easy-1. Miniprep+pac I checking (Fig. 37 B)
8. Chose #2, retransform XL-I Blue, Miniprep, +pac I; Midiprep, +pac I checking (Fig. 37 A)

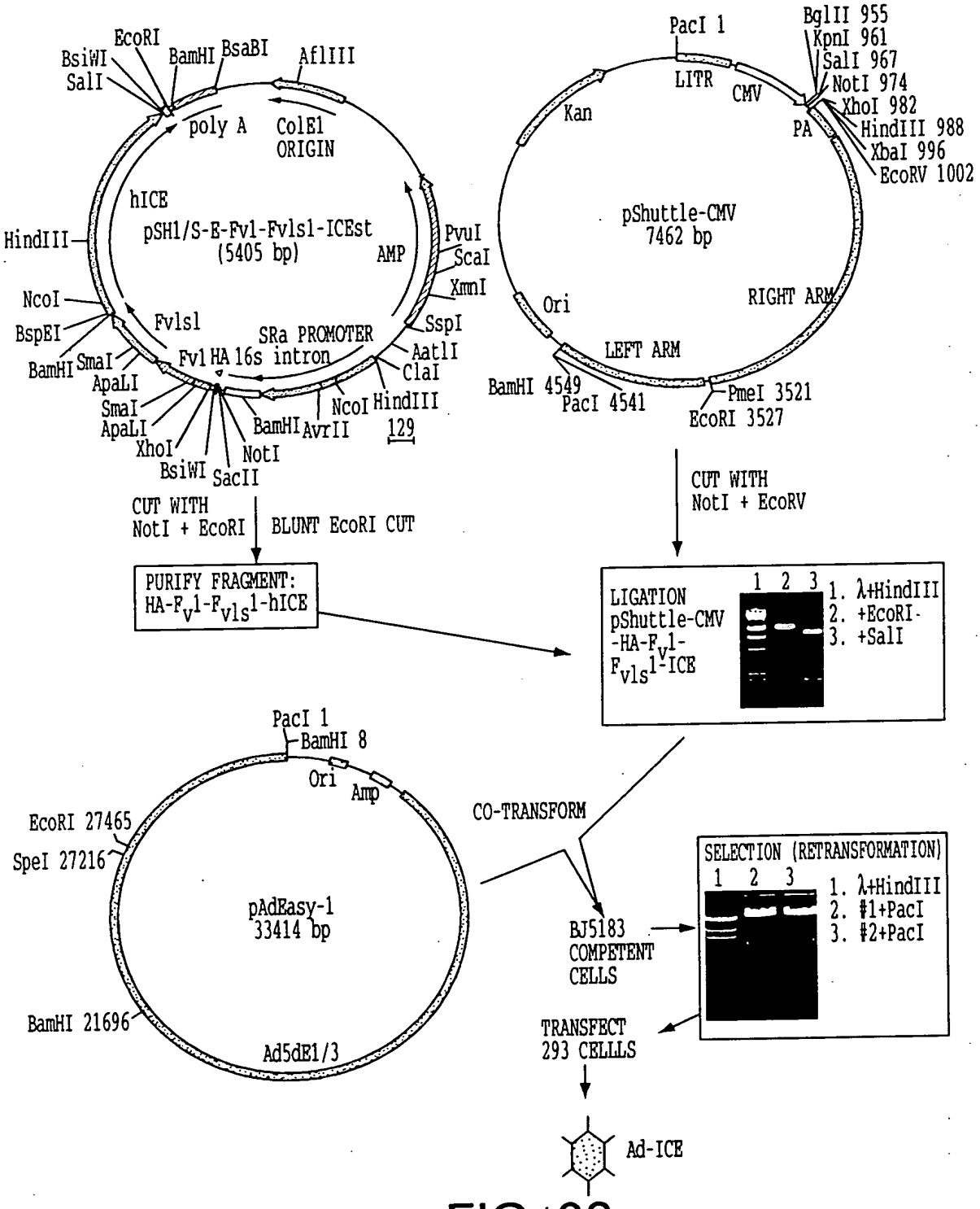

EcoRI
BsiWI
SalI
BamHI
BsaBI
AflIII
poly A
ColE1
ORIGIN
hICE
pSH1/S-E-Fv1-Fvls1-ICEst
(5405 bp)
AMP
HindIII
PvuI
ScaI
XmnI
NcoI
BspEI
BamHI
SmaI
ApaLI
SmaI
ApaLI
XhoI
BsiWI
SacII
NotI
BamHI
AvrII
NcoI
HindIII
129
Fvls1
SRa PROMOTER
Fv1 HA 16s intron
AatII
ClaI
CUT WITH
NotI + EcoRI
BLUNT EcoRI CUT
PURIFY FRAGMENT:
HA-Fv1-Fvls1-hICE
PacI 1
BglII 955
KpnI 961
SalI 967
NotI 974
XhoI 982
HindIII 988
XbaI 996
EcoRV 1002
LITR
CMV
PA
Kan
pShuttle-CMV
7462 bp
Ori
RIGHT ARM
LEFT ARM
BamHI 4549
PacI 4541
PmeI 3521
EcoRI 3527
CUT WITH
NotI + EcoRV
LIGATION
pShuttle-CMV
-HA-Fv1-
Fvls1-ICE
1 2 3
1. λ+HindIII
2. +EcoRI-
3. +SalI
PacI 1
BamHI 8
Ori
Amp
EcoRI 27465
SpeI 27216
pAdEasy-1
33414 bp
BamHI 21696
Ad5dE1/3
CO-TRANSFORM
BJ5183
COMPETENT
CELLS
SELECTION (RETRANSFORMATION)
1 2 3
1. λ+HindIII
2. #1+PacI
3. #2+PacI
TRANSFECT
293 CELLLS
Ad-ICE

FIG. 38

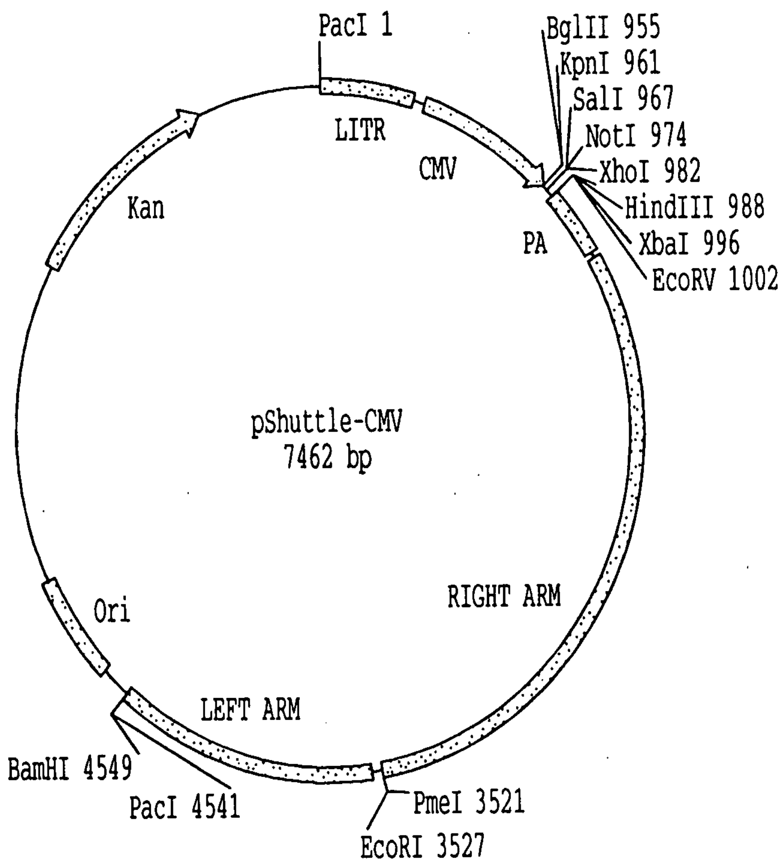


FIG. 39A

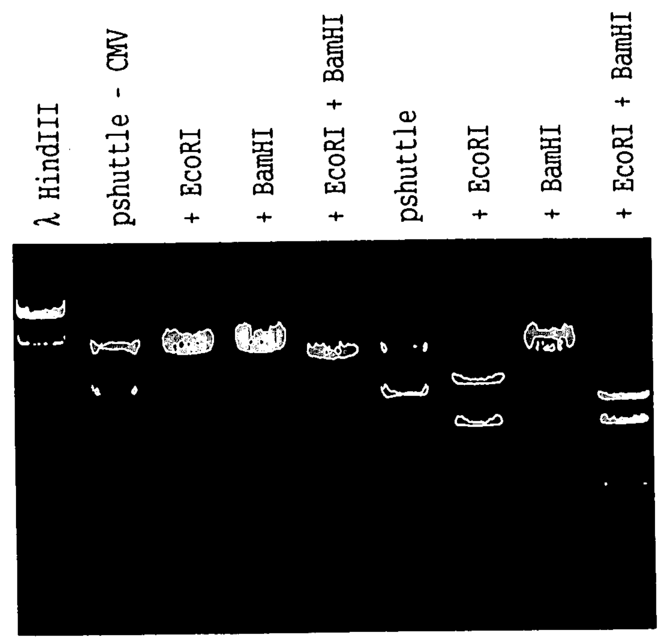


FIG. 39B

Luciferase Assay To Determine the Effect of Different Plasmids

1. Cells were plated in 6 well-plate, $1X10^5$/ well in 3 ml media (RPMI 1640 for JD-2α cells, DMEM for 293 and 293-Z4 cells) with 5% FBS, and incubated for 24 hours.
2. Cells were transfected with 2 μg DNA each (pGL2, pTrack-ICE, pTrack-YAMA, pShuttle-ICE) and FuGene(1 μg DNA/2 μl) and incubated overnight.
3. AP1903, or FK 1012,or AP20187 was added at a final concentration of 50 nM and cultures were incubated 24 hours.
4. Cells were lysed and checked for luciferase activity on a luminometer.

8 JAN 99 AM 10:58 V. 1.1

MEAS. TIMES (S) : 15.0

SAMPLE			RLU	% C.V.	
	1	1	294		
	1	2	291		
MEAN			292	0.8	Control
	2	1	2681958		
	2	2	2769999		
MEAN			2725979	2.3	Control + pGL_2
	3	1	208762		
	3	2	223711		
MEAN			216237	4.9	pGL_2 + ICE
	4	1	22084		
	4	2	22859		
MEAN			22471	2.4	pGL_2 + ICE + AP1903

Figure 40

Effect of Ad-GFP-Ice and YM on Prostate Cancer and BPH Smooth muscle cells
JD-2α, T-C_2, T-C2G, LNCaP-Adeno-YM or Adeno-ICE

1. Plating cells: 24 well-plate
JD-2a -- 20000/well in 1ml RPMI 1640 Media (5%FBS)
T-C_2 -- 10000/well in 1 ml DMEM Media (+5% FBS. 5%Nuserum, Insulin DHT)
T-C_2G -- 10000/well in 1 ml DMEM Media (+5% FBS. 5%Nuserum, Insulin DHT)
LNCaP–30000/well in 1ml RPMI 1640 Media (5%FBS)

incubate JD 24 hrs	cell number doubling - 40000/well
T-C_2, FC_2G-48 hrs.	- 40000/well
LNCaP-72 hrs	- 60000/well

2. Infection:

For JD: ICE 1x10^7 iu/ml	MOI	1	2	4	8	16	
	Add	4	8	16	32	65	μl
YM 2x10^7 iu/ml	MOI	2.5	5	10	20	40	
		5	10	20	40	80	μl
For T-C_2 ICE 5x10^7 iu/ml	MOI	2.5	5	10	20	40	
+ T-C_2G	add	2	4	8	16	32	μl
YM 5x10^7 iu/ml	MOI	2.5	5	10	20	40	
	Add	2	4	8	16	32	μl
YM ICE 5x10^7 iu/ ml	MOI	2.5	5	10	20	40	
For LN Cap		3	6	12	24	48	μl

After 4 hrs. Add AP 1903 →50nM. Incubate 24 hrs.

3. Fix with 1% Glutaraldehyde - PBS, 15';
Stain 0.5% crystal violate, 20'
Wash with H_2O, 30';
Resolve with Sortson's Solution 200 μl/well, 5';
Transfer 100 μl to each well of 96-well plate, Read OD at 570 nM.

Figure 41

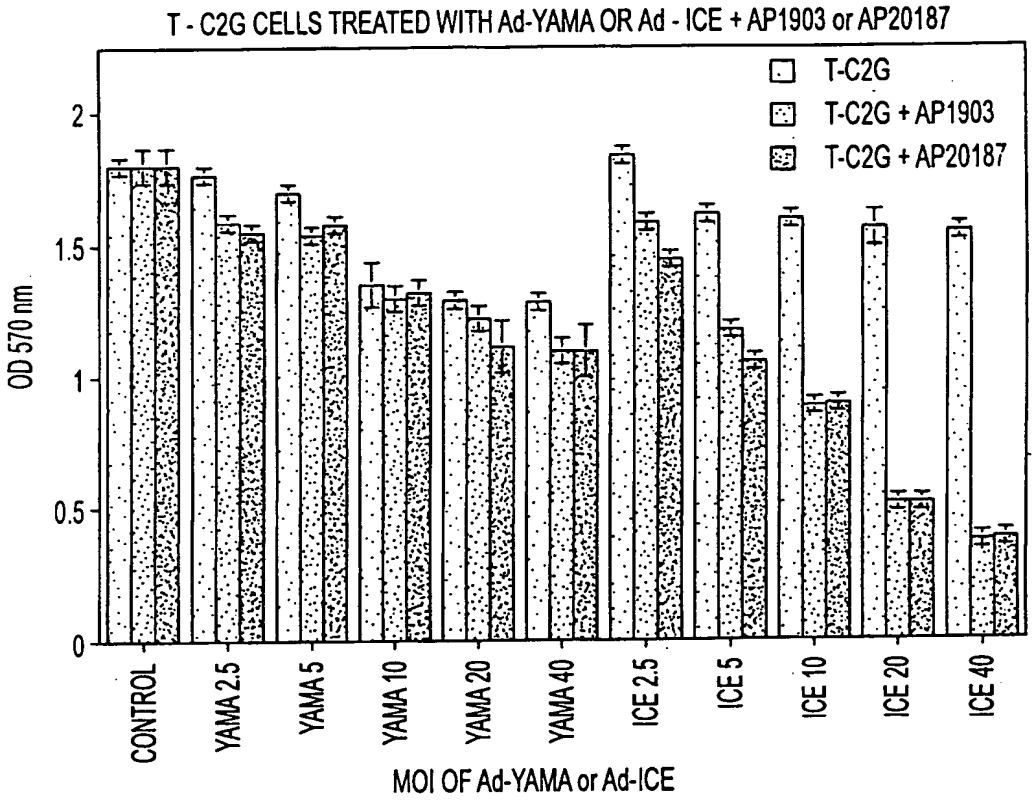

T - C2G CELLS TREATED WITH Ad-YAMA OR Ad - ICE + AP1903 or AP20187
T-C2G
T-C2G + AP1903
T-C2G + AP20187
OD 570 nm
2
1.5
1
0.5
0
CONTROL
YAMA 2.5
YAMA 5
YAMA 10
YAMA 20
YAMA 40
ICE 2.5
ICE 5
ICE 10
ICE 20
ICE 40
MOI OF Ad-YAMA or Ad-ICE

FIG. 42

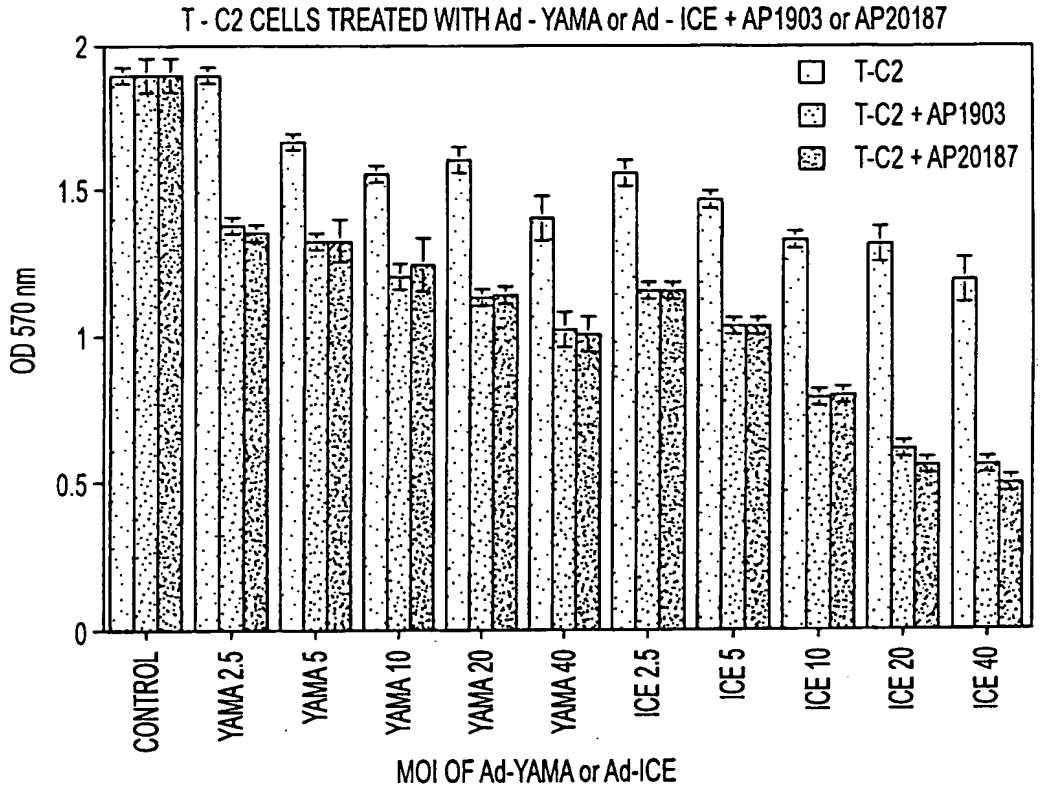

T - C2 CELLS TREATED WITH Ad - YAMA or Ad - ICE + AP1903 or AP20187
T-C2
T-C2 + AP1903
T-C2 + AP20187
OD 570 nm
2
1.5
1
0.5
0
CONTROL
YAMA 2.5
YAMA 5
YAMA 10
YAMA 20
YAMA 40
ICE 2.5
ICE 5
ICE 10
ICE 20
ICE 40
MOI OF Ad-YAMA or Ad-ICE

FIG. 43

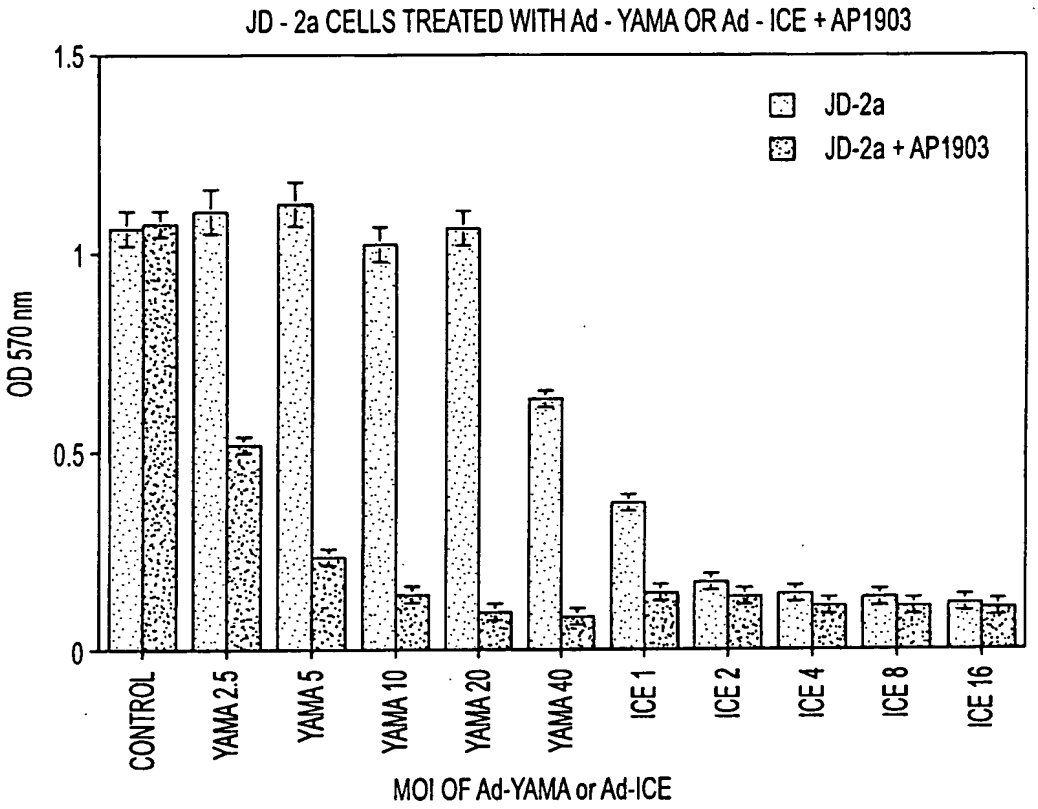


FIG. 44

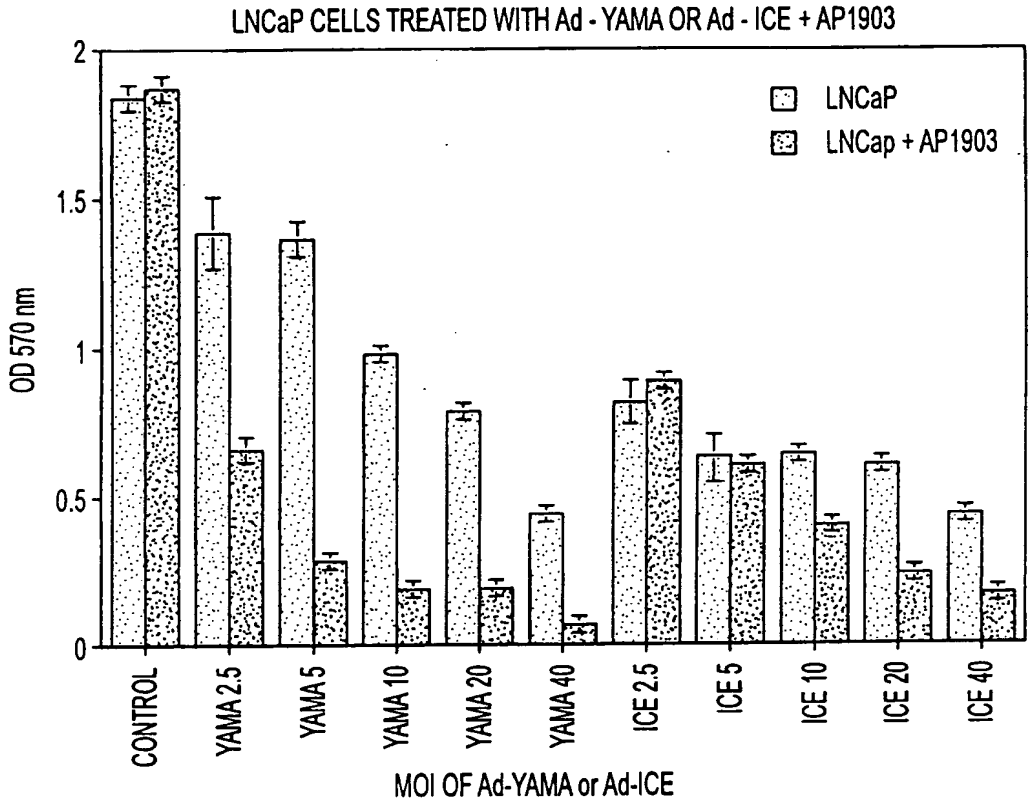

LNCaP CELLS TREATED WITH Ad - YAMA OR Ad - ICE + AP1903
LNCaP
LNCap + AP1903
OD 570 nm
2
1.5
1
0.5
0
CONTROL
YAMA 2.5
YAMA 5
YAMA 10
YAMA 20
YAMA 40
ICE 2.5
ICE 5
ICE 10
ICE 20
ICE 40
MOI OF Ad-YAMA or Ad-ICE

FIG. 45

Western Blot 1103-110498

293 wells; infected with Ad-YM or Ad-ICE
9% Resolving gel; tranfer -- 400mA, 2hrs

1:1000 α-HA11 -- 1st Ab.

1.	Ad-YM3	
2.	"" ""	+1903(100nM)
3.	"" ""	+2D-DCB (100nM)
4.	"" ""	+Both
5.	Ad-ICE	
6.	"" ""	+1903
7.	"" ""	+2-D-DCB
8.	"" ""	+ Both
9.	Ad-YM4	
10.	"" ""	+1903
11.	"" ""	+2-D-DCB
12.	"" ""	+Both

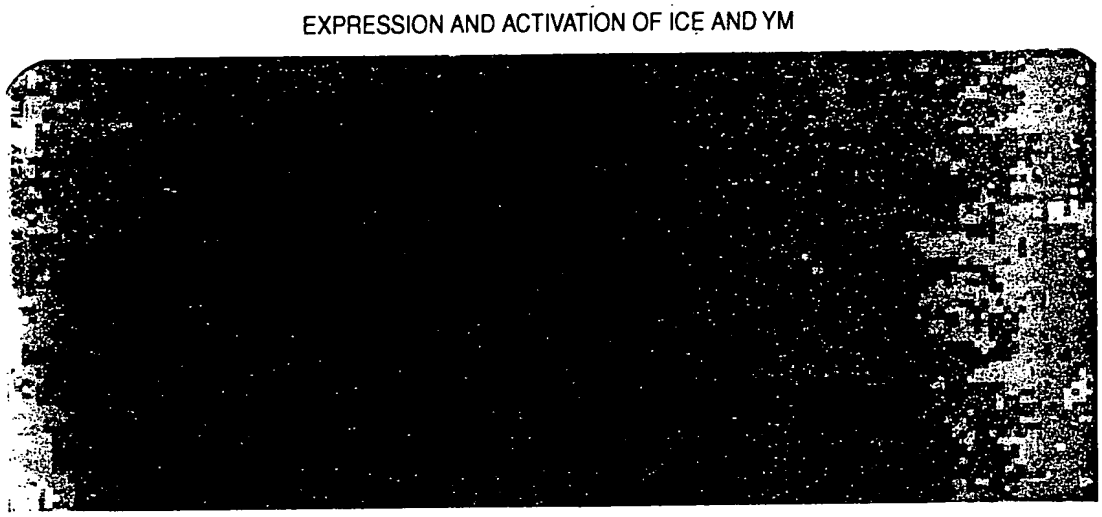


Figure 46

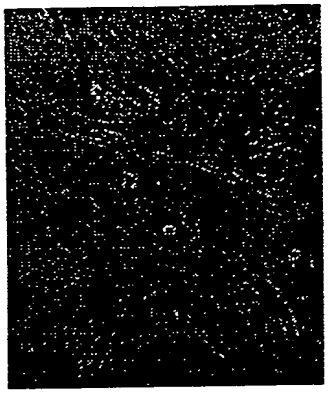


FIG. 47A

FIG. 47B

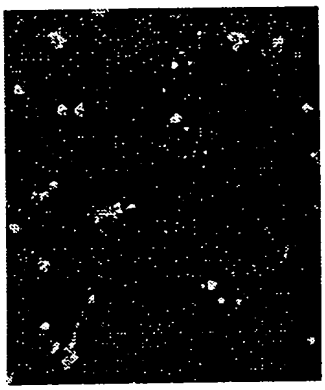


FIG. 47C

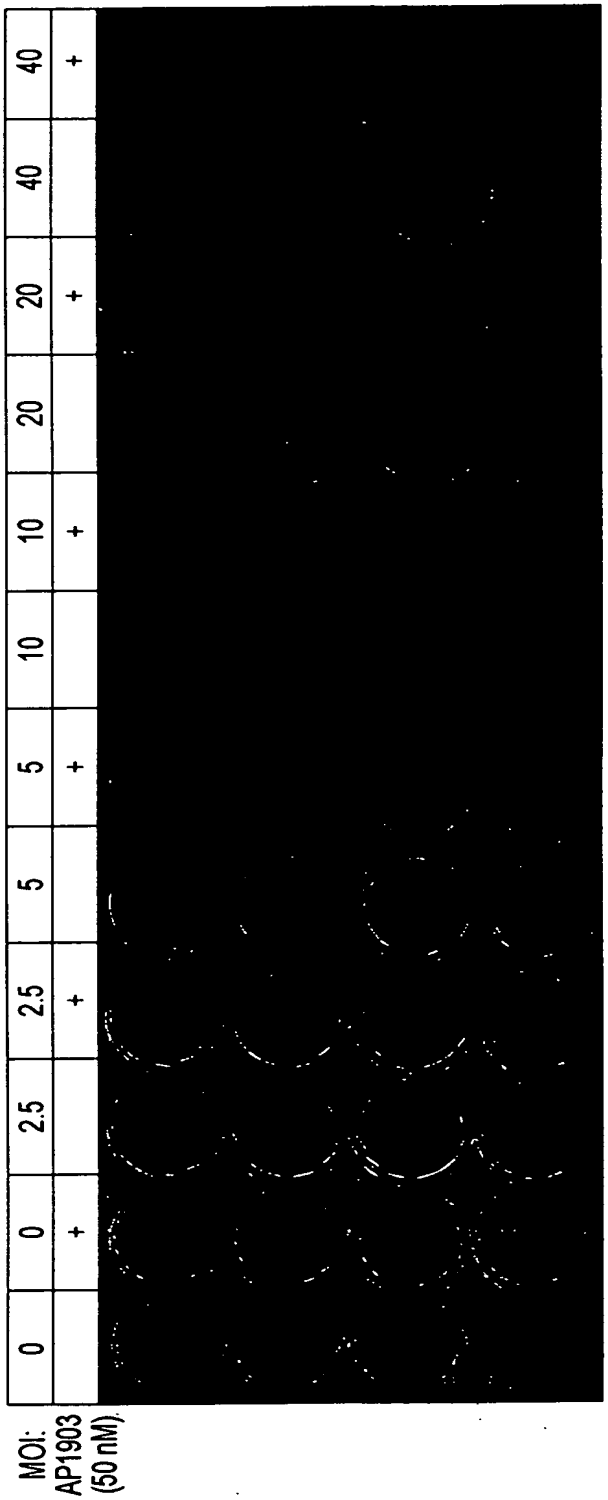


FIG. 48

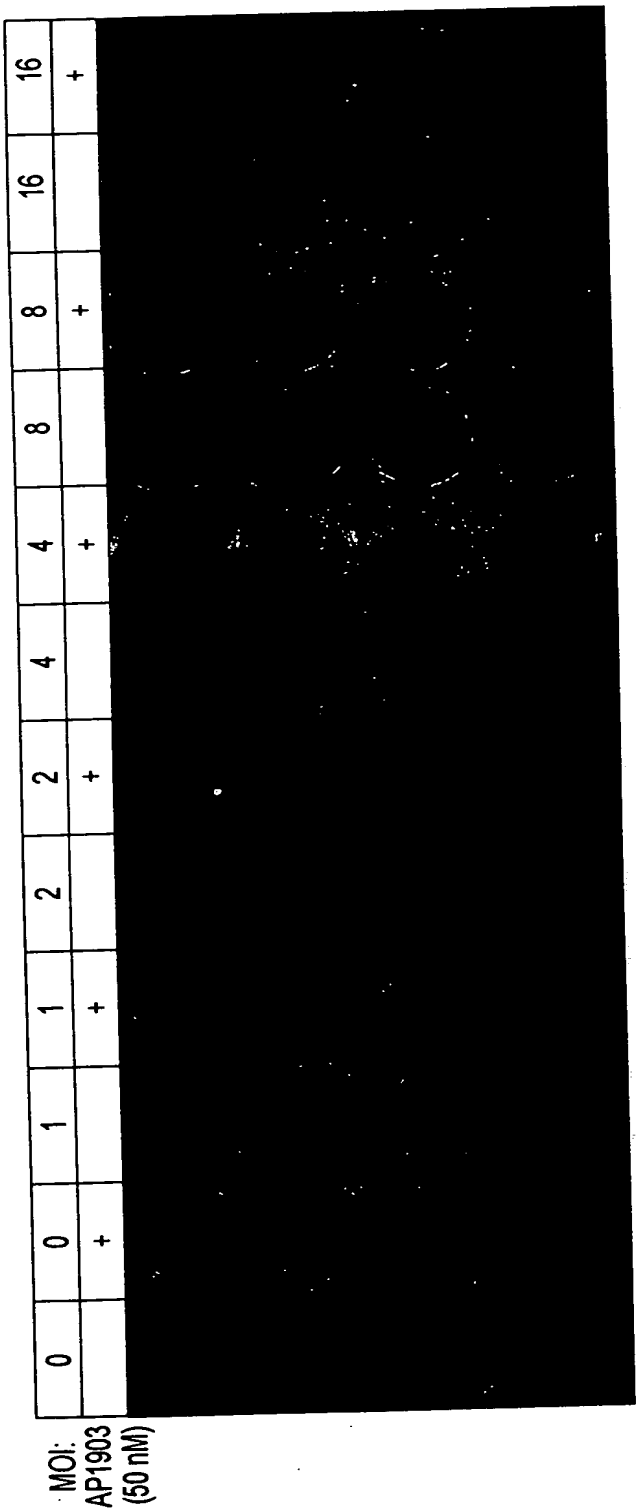


FIG. 49

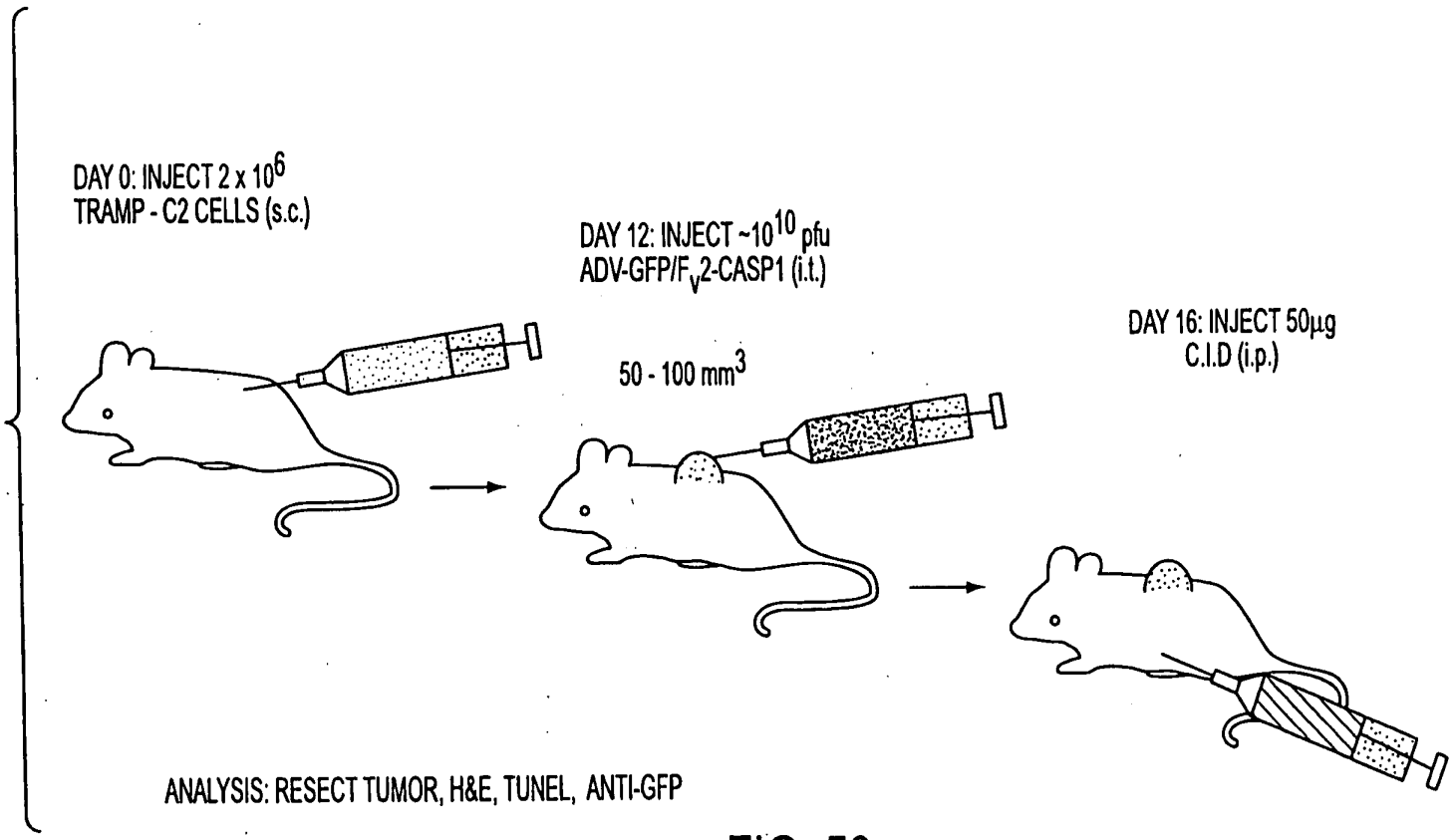


FIG. 50

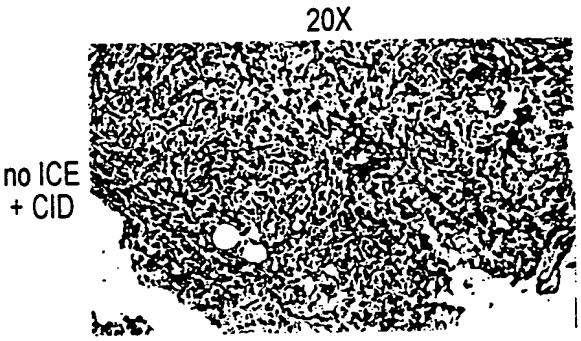


FIG. 51A

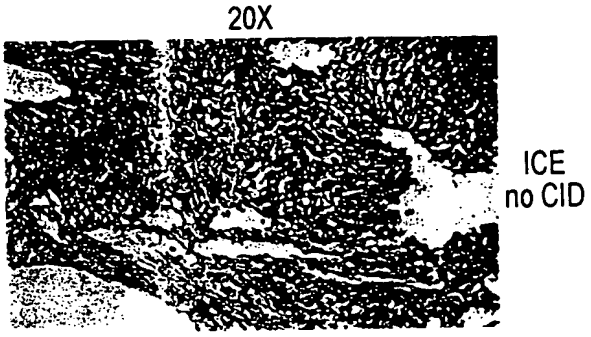


FIG. 51B

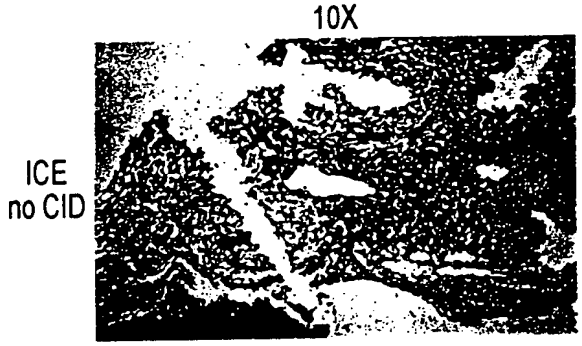


FIG. 51C

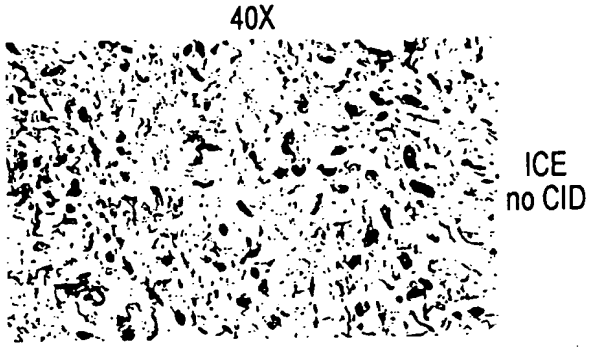


FIG. 51D

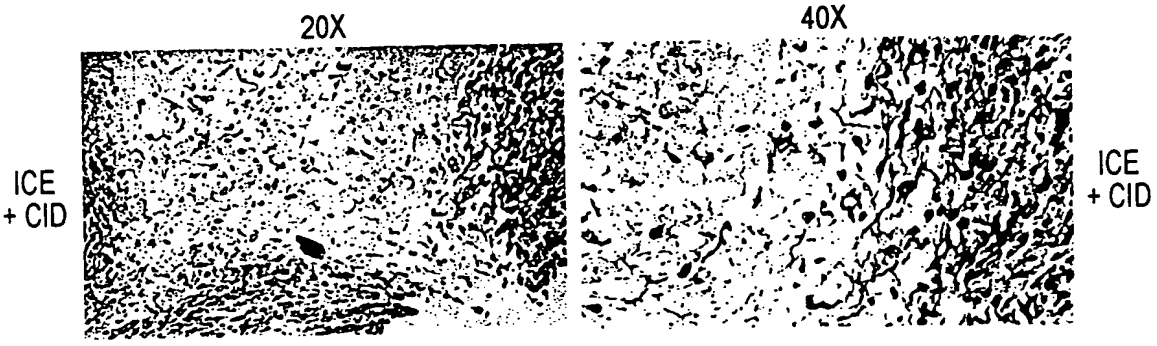


FIG. 52A

FIG. 52B

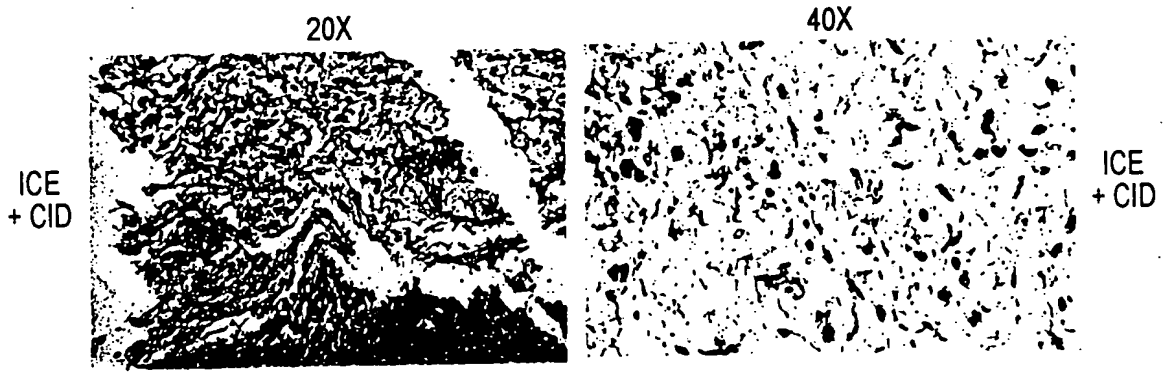


FIG. 52C

FIG. 52D